JUNE 1959, VOLUME 72, No. 2

THE BIOCHEMICAL JOURNAL

UNIVERSITY OF MICHIGAN
JUN 29 1959
SCIENCE LIBRARY

Edited for

THE BIOCHEMICAL SOCIETY

Editorial Board

A. G. OGSTON (*Chairman*) — H. J. ROGERS (*Deputy Chairman*)
E. P. ABRAHAM — D. C. HARRISON
W. N. ALDRIDGE — J. LASCELLES
W. BARTLEY — L. W. MAPSON
E. A. DAWES — R. MARKHAM
R. M. C. DAWSON — R. J. PENNINGTON
G. E. FRANCIS — J. J. SCOTT
Q. H. GIBSON — W. V. THORPE
C. H. GRAY — W. J. WHELAN

Secretary to the Board: F. CLARK

CAMBRIDGE UNIVERSITY PRESS
BENTLEY HOUSE, 200 EUSTON ROAD, LONDON, N.W. 1
AMERICAN BRANCH: 32 EAST 57TH STREET, NEW YORK 22, N.Y.

Price 25s. net (U.S.A. $4.25)

The *Biochemical Journal* is conducted by the **Biochemical Society** and is published by the Cambridge University Press. The *Journal* is issued in three volumes per year. Each volume consists of approximately 700 pages, published in four parts at intervals of one month. The index for each volume is issued separately.

The Biochemical Society was instituted solely for the advancement of the Science of Biochemistry, and holds meetings at different centres for the communication of original papers, and for the discussion of current problems. Persons interested in Biochemistry are eligible for election. Members whose subscriptions are not in arrears are entitled to receive the *Biochemical Journal* without further payment. Further information may be obtained from the Honorary Secretaries, Dr C. E. Dalgliesh, Postgraduate Medical School of London, Ducane Road, Hammersmith, London, W. 12, and Dr P. N. Campbell, The Courtauld Institute of Biochemistry, Middlesex Hospital, London, W. 1, or from the Honorary Treasurer, Dr F. A. Robinson, c/o Allen and Hanburys, Ltd., Bethnal Green, London, E. 2.

Subscriptions to the *Biochemical Journal*. For non-members of the Biochemical Society the subscription to the *Biochemical Journal* is £4 per volume, payable in advance to the Cambridge University Press, Bentley House, 200 Euston Road, London, N.W. 1, or to any bookseller. In the United States of America the *Journal* may be purchased from the Cambridge University Press American Branch, 32 East 57th Street, New York, 22 (subscription price $13.50 per volume).

Communications respecting the sale of single issues or back numbers of the *Journal*, Volumes 20–71, should be addressed to the Cambridge University Press, Bentley House, 200 Euston Road, London, N.W. 1, from whom a list of prices is available. Volumes 1–15 have been reproduced and are available from William Dawson & Sons, Ltd., 4 Duke Street, Manchester Square, London, W. 1.

Claims for the replacement of *Journals* lost in transmission will not be entertained if they are received later than three months after the date of the posting of the *Journal*.

The following are available in paper covers:

Volumes 20–28.
Volume 29 Nos. 1, 2, 4, 6, 8–11.
Volume 33 Nos. 8–12, Index.
Volume 34 Nos. 4–7 & 12.
Volume 34 Nos. 8 & 9, 10 & 11.
Volumes 35–38.
Volume 39 Nos. 2 & 3.
Volumes 40–47.
Volumes 50–58.
Volumes 60–71.
Index to Volumes 11–20.
Index to Volumes 21–30.
Index to Volumes 31–40.
Index to Volumes 41–65.

History of the Biochemical Society.

Reduced terms for Volumes 1–15 are available to members of the Biochemical Society, who should apply to the Honorary Secretaries.

Stu

Ha
Th

Ac
An
A r
Th

Th

Th

En

Cer
Cer
Stu

Mo

Th

Deg

Fac

A c

Thy
The

The
The
Stu

Olig

Fur

The
The

The

CONTENTS OF VOLUME 72, No. 2, 1959

(*All rights reserved*)

PAGE

Studies in detoxication. 79. The metabolism of *cyclo*[^{14}C]hexane and its derivatives. By T. H. Elliott, D. V. Parke and R. T. Williams 193

Haemorrhage and tissue electrolytes. By E. M. Widdowson and D. A. T. Southgate 200

The effect of chlorpromazine on the respiratory chain. Cytochrome oxidase. By M. J. R. Dawkins, J. D. Judah and K. R. Rees 204

Acetylcholinesterase in goldfish muscles. Studies on some substrates and inhibitors. By S. J. Lundin . 210

An unusual property of thyroxine and other iodophenols. By J. R. Tata 214

A new aspect of the interaction between thyroxine and proteins. By J. R. Tata 222

The translocation of antibiotics in higher plants. 3. The estimation of griseofulvin relatives in plant tissue. By S. H. Crowdy, A. P. Green, J. F. Grove, P. McCloskey and A. Morrison 230

The translocation of antibiotics in higher plants. 4. Systemic fungicidal activity and chemical structure in griseofulvin relatives. By S. H. Crowdy, J. F. Grove and P. McCloskey 241

The metabolism of glycerol in *Mycobacterium butyricum*. By N. L. Edson, G. J. E. Hunter, R. G. Kulka and D. E. Wright 249

Enzymic studies of X-irradiated cornea and lens with special reference to glutathione reductase. By R. E. Kuhlman and R. A. Resnik 261

Cerebral metabolism in the sheep. 1. Normal sheep. By B. P. Setchell 265

Cerebral metabolism in the sheep. 2. Irreversible hypoglycaemic coma. By B. P. Setchell 275

Studies on phospholipids. 5. The separation of dinitrophenylated and methylated phospholipids by countercurrent distribution. By F. D. Collins 281

Molecular structure of glycogens isolated from the livers of foetal animals. By F. L. Warren and M. Whittaker 288

The occurrence of methylated guanines in ribonucleic acids from several sources. By J. D. Smith and D. B. Dunn 294

Degradation of the hyaluronic acid complex of synovial fluid by proteolytic enzymes and by ethylenediaminetetra-acetic acid. By A. G. Ogston and T. F. Sherman 301

Factors influencing the incorporation of amino acid into the protein of microsome and mitochondria preparations of rat liver and liver tumour. By O. Greengard and P. N. Campbell 305

A quantitative method for the separation of thyroid hormones and related compounds from serum and tissues with an anion-exchange resin. By V. A. Galton and R. Pitt-Rivers 310

Thyroid-hormone metabolism in the kidney. By V. A. Galton and R. Pitt-Rivers 314

The identification of the acetic acid analogues of thyroxine and tri-iodothyronine in mammalian tissues. By V. A. Galton and R. Pitt-Rivers 319

The biosynthesis of polyacetylenes. 2. Origin of the carbon atoms. By J. D. Bu'Lock and H. Gregory 322

The amide and carboxyl groups of bacitracin A. By D. L. Swallow and E. P. Abraham 326

Studies in the biochemistry of micro-organisms. 106. Metabolites of *Alternaria tenuis* auct.: the structure of tenuazonic acid. By C. E. Stickings 332

Oligosaccharide synthesis in the banana and its relationship to the transferase activity of invertase. By R. W. Henderson, R. K. Morton and W. A. Rawlinson 340

Fungal detoxication. 4. Metabolism of 2-methoxynaphthalene by *Aspergillus niger*. By R. J. W. Byrde, D. F. Downing and D. Woodcock 344

The mechanism of ficin-catalysed reactions. By B. R. Hammond and H. Gutfreund 349

The effects of adenosine triphosphate on the mechanical properties of surface films of L-myosin and actomyosin. By D. F. Cheesman, M. M. Keeler and O. Sten-Knudsen 357

The copper content and cytochrome oxidase activity of tissues from normal and swayback lambs. By J. McC. Howell and A. N. Davison 365

Proceedings of the Biochemical Society

PAGE

Ultraviolet fluorescence of proteins. By F. W. J. Teale and G. Weber 15P

Polarization of the ultraviolet fluorescence and electronic energy transfer in proteins. By G. Weber and F. W. J. Teale 15P

On the intramolecular location of deoxyribonucleotides incorporated into deoxyribonucleic acid by extracts of ascites tumour cells. By E. D. Gray and R. M. S. Smellie 16P

Net synthesis of deoxyribonucleic acid by extracts of ascites tumour cells. By R. M. S. Smellie, H. M. Keir, E. D. Gray, D. Bell, J. Richards and J. N. Davidson 17P

Enzymic formation of deoxyribonucleoside triphosphates. By H. M. Keir 17P

Patterns of oxidative assimilation in *Sarcina lutea*. By B. Binnie, W. H. Holms and E. A. Dawes . 17P

Interaction between pyridoxal phosphate and amino acids. By H. K. King and N. Lucas . . . 18P

Aryl sulphamate synthesis. By A. B. Roy 19P

Photosynthetic uptake of labelled carbon dioxide by sub-cellular components of spinach leaves. By V. Moses, O. Holm-Hansen, N. G. Pon, K. Nishida and M. Calvin 19P

The possible conversion of fat into carbohydrate in the obese human. By G. L. S. Pawan . . . 20P

Stimulation of liver acid phosphatase activity following the parenteral injection of excessive doses of iron. By L. Golberg, L. E. Martin, C. M. Bates and A. Batchelor 20P

Succinyl group-translocation through the plasma membrane of *Micrococcus lysodeikticus*. By P. Mitchell and J. Moyle 21P

The effect of isoniazid, propamidine, pentamidine and carbutamide on the growth and transaminases of *Escherichia coli*. By R. M. Hicks 22P

Purification of growth hormone from pig pituitaries. By J. H. Ottaway 22P

Purification and properties of a proteinase from *Bacillus cereus* NCTC 945. By D. N. Salter . . 23P

Hypersensitivity of the visual cell to iodoacetate. By C. N. Graymore 23P

Amino acid incorporation into proteins of isolated rat-liver mitochondria. By P. J. Reis, J. L. Coote and T. S. Work 24P

An apparatus for incubating tissue slices *in vitro*. By J. H. Ottaway, A. K. Huggins and J. Martin . 25P

INDEX OF AUTHORS

	PAGE
Abraham, E. P.	326
Batchelor, A.	20P
Bates, C. M.	20P
Bell, D.	17P
Binnie, B.	17P
Bu'Lock, J. D.	322
Byrde, R. J. W.	344
Calvin, M.	19P
Campbell, P. N.	305
Cheesman, D. F.	357
Collins, F. D.	281
Coote, J. L.	24P
Crowdy, S. H.	230, 241
Davidson, J. N.	17P
Davison, A. N.	365
Dawes, E. A.	17P
Dawkins, M. J. R.	204
Downing, D. F.	344
Dunn, D. B.	294
Edson, N. L.	249
Elliott, T. H.	193
Galton, V. A.	310, 314, 319
Golberg, L.	20P
Gray, E. D.	16P, 17P
Graymore, C. N.	23P
Green, A. P.	230
Greengard, O.	305
Gregory, H.	322
Grove, J. F.	230, 241
Gutfreund, H.	349
Hammond, B. R.	349
Henderson, R. W.	340
Hicks, R. M.	22P
Holm-Hansen, O.	19P
Holms, W. H.	17P
Howell, J. McC.	365
Huggins, A. K.	25P
Hunter, G. J. E.	249
Judah, J. D.	204
Keeler, M. M.	357
Keir, H. M.	17P
King, H. K.	18P
Kuhlman, R. E.	261
Kulka, R. G.	249
Lucas, N.	18P
Lundin, S. J.	210
McCloskey, P.	230, 241
Martin, J.	25P
Martin, L. E.	20P
Mitchell, P.	21P
Morrison, A.	230
Morton, R. K.	340
Moses, V.	19P
Moyle, J.	21P
Nishida, K.	19P
Ogston, A. G.	301
Ottaway, J. H.	22P, 25P
Parke, D. V.	193
Pawan, G. L. S.	20P
Pitt-Rivers, R.	310, 314, 319
Pon, N. G.	19P
Rawlinson, W. A.	340
Rees, K. R.	204
Reis, P. J.	24P
Resnik, R. A.	261
Richards, J.	17P
Roy, A. B.	19P
Salter, D. N.	23P
Setchell, B. P.	265, 275
Sherman, T. F.	301
Smellie, R. M. S.	16P, 17P
Smith, J. D.	294
Southgate, D. A. T.	200
Sten-Knudsen, O.	357
Stickings, C. E.	332
Swallow, D. L.	326
Tata, J. R.	214, 222
Teale, F. W. J.	15P
Warren, F. L.	288
Weber, G.	15P
Whittaker, M.	288
Widdowson, E. M.	200
Williams, R. T.	193
Woodcock, D.	344
Work, T. S.	24P
Wright, D. E.	249

FORTHCOMING PAPERS

It is hoped to publish the following papers in the next issue of the *Biochemical Journal:*

Hydroxystilbenes of *Eucalyptus wandoo.* By D. E. Hathway and J. W. T. Seakins

A study of vitamin B_{12} protection in experimental thyrotoxicosis in the rat. By D. K. Kasbekar, W. V. Lavate, D. V. Rege and A. Sreenivasan

A study of vitamin B_{12} protection in experimental liver injury to the rat by carbon tetrachloride. By D. K. Kasbekar, W. V. Lavate, D. V. Rege and A. Sreenivasan

Biosynthesis of glutathione by rat erythrocytes. By D. K. Kasbekar and A. Sreenivasan

Biosynthesis of diaminopimelic acid and lysine in *Escherichia coli:*

1. The incorporation of ^{14}C from various organic precursors into the diaminopimelic acid of a lysine-requiring mutant. By Pauline Meadow and Elizabeth Work

2. Incorporation of [^{14}C]diaminopimelic acid, lysine and glucose. By Pauline Meadow and Elizabeth Work

The estimation of anserinase activity by a low-temperature ninhydrin reaction. By N. R. Jones

The metabolism of acetaldehyde in mammalian tissues. Reactions in rat-liver suspensions under anaerobic conditions. By F. Lundquist, Ulla Fugmann, Edith Kläning and H. Rasmussen

A ribonucleoprotein component of *Escherichia coli.* By T. J. Bowen, S. Dagley and J. Sykes

The oxidative pathway of carbohydrate metabolism in *Escherichia coli.* 6. Adaptation of glucose 6-phosphate dehydrogenase to growth in complex media. By Dwight B. McNair Scott and Eileen Chu

Effect of ethanol on rat-liver ribonucleoprotein previously exposed to cold trichloroacetic acid. By P. R. Venkataraman and C. U. Lowe

The role of cytoplasmic ribonucleic acid in the incorporation of amino acids into microsomal proteins. By R. Rendi and P. N. Campbell

Investigation of the products of metabolism after incubation of deoxycorticosterone with rabbit-liver homogenate. By W. Taylor

The metabolism of 2:4:5-trihydroxybutyrophenone in the rat and dog. By B. D. Astill, D. W. Fassett and R. L. Roudabush

Metabolism of the developing retina. 2. The effect of an inherited retinal degeneration on the development of glycolysis in the rat retina. By C. N. Graymore, Katharine Tansley and Margaret Kerly

Some effects of salicylate on mitochondria from rat liver. By S. W. Jeffrey and M. J. H. Smith

Some cation-binding properties of cartilage. By J. R. Dunstone

The acute toxic action of dimethylnitrosamine on liver cells. By Margot J. Bailie and G. S. Christie

Identification of amino sugars. By M. J. Crumpton

The relation between the activity of a lecithinase and the electrophoretic charge of the substrate. By A. D. Bangham and R. M. C. Dawson

The activation of surface films of lecithin by amphipathic molecules. By R. M. C. Dawson and A. D. Bangham

Studies on the metabolism of hydroxypyruvate in animal tissues. By F. Dickens and D. H. Williamson

Adaptation to form bacteriochlorophyll in *Rhodopseudomonas spheroides*: changes in activity of enzymes concerned in pyrrole synthesis. By June Lascelles

The effect of ionizing radiation on nuclear phosphorylation in the radio-sensitive tissues of the rat. By W. A. Creasey and L. A. Stocken

The aerobic inhibition of glycolysis in pea-seed extracts and its possible relationship to the Pasteur effect. By M. D. Hatch and J. F. Turner

Studies on phospholipids. 6. A complex phospholipid from sheep brain. By F. D. Collins

A study of the proteinase content and the chromatography of thymus histones. By D. M. P. Phillips and E. W. Johns

THE BIOCHEMICAL JOURNAL

NOTES FOR CONTRIBUTORS

Papers submitted for publication in the *Biochemical Journal* should be written concisely. The sections below concerning the preparation of the typescript give only general indications. Authors are urged to consult the more detailed *Suggestions to Authors, Chemical Nomenclature and Abbreviations, Symbols, Usages and Conventions,* which was published in May 1957 (*Biochem. J.* 1957, 66, 1); also *Notes on Preparation of Illustrations,* which was published in January 1956 (*Biochem. J.* 1956, 62). Copies of these two pamphlets may be obtained from the Editorial Office (*Biochemical Journal*), The Lister Institute, Chelsea Bridge Road, London, S.W. 1, price 1*s.* 6*d.* (*Suggestions to Authors*) and 1*s.* (*Notes on Preparation of Illustrations*) post free.

Strict observance of the requirements of the *Biochemical Journal* will shorten the period between the receipt of a paper and its publication.

Communications. Papers submitted for publication should be sent to the Secretary to the Editorial Board (*Biochemical Journal*), The Lister Institute of Preventive Medicine, Chelsea Bridge Road, London, S.W. 1.

Abstracts. Authors are requested to submit with their typescript an abstract suitable for inclusion in *International Abstracts of Biological Sciences.* This abstract will not appear in the *Biochemical Journal* but will be edited before being passed for publication in the *Abstracts.*

The abstract should outline as briefly as possible the results and definitive conclusions of the work submitted. Details of methods are generally not required. A paper of average length should be abstracted in about 100 words. The abstract should be typed in double spacing on a separate quarto sheet in the following form: title; name(s) of author(s); *Biochem. J.* (space for year, volume and page reference); address (for reprint applications); abstract. For example:

> The metabolism of short-chain fatty acids in the sheep. 4. The pathway of propionate metabolism in rumen epithelial tissue. R. J. Pennington and T. M. Sutherland. *Biochem. J.* 1956, **63**, 618–628 (Rowett Research Institute, Bucksburn, Aberdeenshire, Scotland)—(Abstract).

Reprints. Where at least one author of a paper is a member of the Biochemical Society, twenty-five reprints are supplied free of cost. An author may purchase additional reprints if he notifies the Press on the appropriate form immediately the proof of the paper is received. Communications about the purchase of reprints should be addressed to the University Press, Cambridge.

General. It is the policy of the *Biochemical Journal* to publish papers in all fields of biochemistry—plant, animal and microbiological—provided that they describe results which make a new and fundamental contribution to biochemical knowledge, or that they describe methods applicable to biochemical problems. Submission of a paper to the Editorial Board will be held to imply that it reports unpublished work, that it is not under consideration for publication elsewhere, and that if accepted for the *Biochemical Journal* it will not be published elsewhere in the same form, either in English or in any other language, without the consent of the Editorial Board.

Contributors who reside abroad may nominate somebody in Great Britain who is willing to correct the proofs of their papers. Proofs are also sent to all authors residing abroad, if necessary by airmail, whether or not they have nominated a proof reader

p.t.o.

in Great Britain; if these are returned immediately, it will normally be possible to incorporate corrections in the final proof. The method of correcting proofs given in B.S. 1219 or B.S. 1219C (obtainable from the British Standards Institution) is preferred.

Papers should be headed by an informative title, by the names of the authors and by the name and address of the Laboratory where the work was performed. Female authors should use one given name and the surname, and male authors should use initials and surname only. Descriptive material about the author, e.g. Beit Memorial Fellow, or details of financial support, should appear as a footnote on the first page or, preferably, in the acknowledgements at the end of the paper.

Typescripts should bear the name and address of the person to whom the proof of the paper is to be sent, and should give also a shortened version of the title, not exceeding forty-five letters and spaces in length, suitable for a running title in the published pages of the work.

If a paper that has been returned to an author for revision is not resubmitted within six months, it will be deemed to be a new paper and the date of receipt altered accordingly. A revised paper containing a significant amount of new material will also be redated.

A paper should be written only when a piece of work is rounded off. Preliminary or abortive experiments should not be described.

It would help the editors if the author, when submitting a paper which is part of a series, would enclose reprints of the immediately preceding parts.

Form of papers submitted for publication. Papers should be in double-spaced typing on sheets of uniform size with wide margins. Top copies only should be submitted. The paper should be written in English.

The onus of preparing a paper in a form suitable for sending to press lies in the first place with the author, who should first consult the detailed *Suggestions to Authors, Chemical Nomenclature and Abbreviations, Symbols, Usages and Conventions.* Authors should also refer to a current issue of the *Journal* in order to make themselves familiar with the typographical conventions, use of cross-headings, lay-out of tables, citation of references, etc. The need for editorial revision of a badly prepared typescript will lead to delay in publication. Papers on specialized subjects should be presented so that they are intelligible to the ordinary reader of the *Journal*. Sufficient information should be included to permit repetition of the experimental work.

Generally, papers should be divided clearly into sections, as follows: (*a*) introduction, containing the reasons for doing the work; (*b*) Experimental methods: with chemical papers the experimental part may appear towards the end, but otherwise should follow the introduction; (*c*) Results: these should be given concisely; the use of both tables and figures to illustrate the same results will only rarely be permitted; only illustrative protocols should be included; (*d*) Discussion: it is desirable that the presentation of the results should be separated from the discussion of their significance; this section should be strictly limited to discussion, and should not recapitulate results; (*e*) a Summary, about 3 % of the length of the paper: the paragraphs of the Summary should be numbered; (*f*) acknowledgements; (*g*) References. The arrangement suggested for sections (*b*)–(*d*) is not binding on authors; other ways of arrangement are sometimes more suitable.

The *Biochemical Journal* uses as a standard for spelling the *Concise Oxford Dictionary of Current English* (Oxford: Clarendon Press).

Illustrations. Diagrams that do not conform with the directions given in *Notes on Preparation of Illustrations* may have to be redrawn by the Press and the expense charged to the author. Legends and captions should be written so that the general meaning of each illustration can be understood without reference to the text, and so that the exact experimental conditions used to obtain the results illustrated are made clear. Illustrations requiring reproduction as half-tone plates should be avoided whenever possible. Photographs or drawings of paper chromatograms, particularly one-dimensional, are not generally published.

Tables. Tables should have headings which make their general meaning comprehensible without reference to the text. Conditions specific to the particular experiment should be stated. Reference to the text for general experimental methods is permissible provided that there is no ambiguity. The units in which the results are expressed, e.g. g./100 ml., should be given at the top of each column, and not repeated on each line of the table.

Tables should be typed on separate sheets and their approximate position in the text indicated. Words or numerals should be repeated on successive lines: 'ditto' or ',,' are not to be used.

Footnotes. These should be avoided in the text as far as possible.

Studies in Detoxication

79. THE METABOLISM OF *cyclo*[^{14}C]HEXANE AND ITS DERIVATIVES*

BY T. H. ELLIOTT, D. V. PARKE AND R. T. WILLIAMS
Department of Biochemistry, St Mary's Hospital Medical School, London, W. 2

(*Received 25 November* 1958)

Little is known about the metabolic transformations of simple alicyclic hydrocarbons such as *cyclo*hexane. In contrast, there is a considerable amount of information about the more complex alicyclic compounds such as the steroids. A knowledge of the fate in the body of *cyclo*hexane and some of its derivatives, such as *cyclo*hexanol and *cyclo*hexanone, could be of value in the study of the metabolism of the more complex compounds. Their widespread use in industry has prompted many studies of their toxic effects on animals (see Browning, 1953), but previous investigations of their metabolic fate have produced little definite information. According to Filippi (1914) *cyclo*hexane is converted into *cyclo*hexanone in rabbits; Torboli (1937) reported that it formed adipic acid. Bernhard (1937) was unable to find any urinary metabolites of *cyclo*hexane in the dog except small amounts of oxalic acid, and presumed that it was totally oxidized in this animal. Treon, Crutchfield & Kitzmiller (1943), however, reported that, in rabbits, conjugated glucuronic acid corresponding to 5% of the dose was excreted after dosage with *cyclo*hexane. Incubation of *cyclo*hexane with ox or sheep kidney or liver is believed to produce small amounts of a substance giving reactions for ketones (Fabre, Truhaut & Peron, 1952); *cyclo*hexane is oxidized by *Pseudomonas aeruginosa* to adipic acid, valeric acid and formic acid, *cyclo*hexyl hydroperoxide being considered as an intermediate (Imelik, 1948).

Bernhard (1937) and Weitzel (1950) found no urinary metabolites of *cyclo*hexanol in dogs, but Treon *et al.* (1943) found that in rabbits this alcohol caused an increased excretion of conjugated glucuronic acid corresponding to 50% of the dose.

Filippi (1914) reported that *cyclo*hexanone yielded small amounts of adipic acid in rabbits, whereas Treon *et al.* (1943) found that it gave rise to the excretion of substantial amounts of conjugated glucuronic acid.

One of the difficulties in investigating the metabolism of *cyclo*hexane is the lack of specific tests for the compound and its metabolites. By using ^{14}C-labelled *cyclo*hexane many of these difficulties can be overcome, and we shall show that, as far as the rabbit is concerned, the main metabolite of *cyclo*hexane, *cyclo*hexanol and *cyclo*hexanone is *cyclo*hexyl glucuronide, which was isolated, and that *cyclo*hexane and *cyclo*hexanol are also hydroxylated to *trans-cyclo*hexane-1:2-diol.

* Part 78: Baldwin, Robinson & Williams (1959).

MATERIALS AND METHODS

Materials. The following compounds were prepared or purchased, and purified (melting points are corrected): *cyclo*hexane (British Drug Houses Ltd.), b.p. 81°, n_D^{20} 1·4262; *cyclo*hexanol (British Drug Houses Ltd.), b.p. 161–162°, n_D^{20} 1·4650; *cyclo*hexanone (Harrington Bros. Ltd.), b.p. 155·5°, n_D^{20} 1·450; *cis-cyclo*hexane-1:2-diol, m.p. 98° (Vogel, 1956); (±)-*trans-cyclo*hexane-1:2-diol, m.p. 103° (Horning, 1955); *cis-* and *trans-cyclo*hexane-1:3-diol were separated from a commercial mixture (L. Light and Co.) according to Clarke & Owen (1950), *cis-*, m.p. 86°, and *trans-*, m.p. 116°; *cis-* and *trans-cyclo*hexane-1:4-diol, m.p. 108–110° and 141° respectively, were the gift of Dr L. N. Owen; *cyclo*hex-1-enyl acetate, b.p. 181° (Machinskaya, 1952); *cyclo*hexane-1:2-dione, m.p. 36° (Arnold, 1952); *trans*-2-amino*cyclo*hexanol, m.p. 65° (Wilson & Read, 1935); *cyclo*pentanealdehyde, b.p. 136° (McCasland, 1951); *cyclo*hexene oxide, b.p. 130° (Gilman & Blatt, 1941); *cyclo*hex-2-enol, b.p. 68°/15 mm. Hg, n_D^{25} 1·4832, and its phenylurethane, m.p. 105·5° (Bartlett & Woods, 1940). The derivatives of the above-mentioned compounds needed for identification and isotopic dilution were prepared and purified by standard methods. All derivatives used have been described in the literature.

cyclo[^{14}C]Hexane was purchased from The Radiochemica Centre, Amersham, Bucks. After a number of biological experiments had been carried out, this material was found to contain a small percentage of [^{14}C]benzene. By conversion into *m*-dinitrobenzene after adding excess of carrier benzene, the [^{14}C]benzene content of the *cyclo*hexane was found to be 1·9%. By conversion into phenol toluene-*p*-sulphonate through sulphonation and alkali-treatment after adding excess of carrier benzene, the [^{14}C]benzene content was found to be 1·5%. One small sample of *cyclo*hexane freed from traces of benzene by gas chromatography was later supplied by The Radiochemical Centre and used in one experiment (see Table 4).

Analytical methods. Glucuronic acid and ethereal sulphate in urine were determined by methods previously described in this series (e.g. Mead, Smith & Williams, 1958).

Paper chromatography. R_P values for the *cyclo*hexane-1:2-diols are shown in Table 1, and agree with those given by Henbest & Wilson (1957). The glucuronides of *cyclo*hexanol and of the *cyclo*hexane-1:2-diols could be separated on paper, but the glucuronides of the *cis-* and *trans*-diol could not.

Table 1. *R_F values of cyclohexanediols and related glucuronides*

R_F values for descending chromatography on Whatman no. 4 paper: A, in butan-2-one–xylene–water (1:1:1, by vol.); B, in propanol–aq. NH_3 soln. (sp.gr. 0·88) (7:3, v/v); C, in butanol–acetic acid–water (4:1:2, by vol.). The *cyclo*hexanediols were detected on paper with ammoniacal silver nitrate solution according to Henbest & Wilson (1957). The glucuronides used were obtained biosynthetically as gums (see text). An aqueous 1% solution (50 μl.) of the glucuronide gums was placed on the paper. They were detected by spraying the paper with a fresh solution of naphtharesorcinol (2% in aqueous 33% trichloroacetic acid), drying at room temperature and then heating at 135° for about 5 min. The glucuronides showed as deep-blue spots, the glucuronide of *cyclo*hexanol giving a more intense colour than the glucuronides of the diols.

Solvent	R_F A	B	C
*trans-cyclo*Hexane-1:2-diol	0·25	—	—
*cis-cyclo*Hexane-1:2-diol	0·38	—	—
*cyclo*Hexyl glucuronide	—	0·55	0·59
*trans-cyclo*Hexane-1:2-diol glucuronide	—	0·46	0·42
*cis-cyclo*Hexane-1:2-diol glucuronide	—	0·43	0·40

Animals and collection of expired air. Adult chinchilla doe rabbits were used and were kept on a constant diet (60 g. of diet 41, Associated Flour Millers Ltd., plus 100 ml. of water daily). Compounds were administered with water by stomach tube. For collection of expired air an animal was housed in a sealed Perspex chamber through which a current of air could be drawn (cf. Parke & Williams, 1950, 1953) and which had an arrangement for collecting urine periodically without breaking the air current. The air after passing through the chamber was led through various Drechsel bottles for trapping CO_2 (in 4N-NaOH), ketones (1% of 2:4-dinitrophenylhydrazine in N-HCl) and hydrocarbons (ethanol at −70°), as described by Parke & Williams (1953). The recovery of *cyclo*hexane from the apparatus was checked with *cyclo*[^{14}C]hexane. Thus in 10 hr. 96% of 0·844 g. of *cyclo*hexane placed on a watch-glass in the chamber could be recovered by aeration into ethanol, the material being estimated from its radioactivity.

Measurement of radioactivity. All measurements were carried out on solid or liquid samples of 'infinite thickness' in an end-window counter. The specific activities were determined by comparison with a stable reference polymer (The Radiochemical Centre) and sufficient counts to give a standard error of ±2% were taken.

Radioactivity in urine was measured by counting the residues obtained by evaporation of the urines on planchets under an infrared lamp or by direct counting of liquid samples. The results by the two procedures did not differ significantly. With tissues and faeces, radioactivity was determined after conversion into $BaCO_3$ by wet oxidation (Van Slyke, Plazin & Weisiger, 1951) and in some cases by direct measurement on tissue homogenates after drying. Radioactive carbon dioxide in expired air was determined in the usual way, as $BaCO_3$, and total hydrocarbons trapped in the ethanol absorption bottles were estimated by direct counting of liquid samples.

cyclo*Hexane in expired air.* Each absorption bottle used to trap *cyclo*hexane contained 20 ml. of ethanol. Carrier *cyclo*hexane (0·8 g.) was added to half of the contents of each bottle. Then an equal volume of water was added, the *cyclo*hexane separated by centrifuging and allowed to stand over sodium for 24 hr. It was then distilled in the Craig microdistillation apparatus, the fraction of b.p. 81° being collected. This was converted into $BaCO_3$ by the wet-oxidation method and counted. *cyclo*Hexanol was determined by adding 1 ml. of carrier to 5 ml. of the ethanolic absorption fluid, the mixture was fractionally distilled and the fraction, b.p. 161–162°, treated with 3:5-dinitrobenzoyl chloride. The *cyclo*hexanol was counted as the dinitrobenzoate. *cyclo*Hexanone was determined by adding 0·5 ml. of carrier to 5 ml. of ethanolic fluid, then isolating the ketone as the 2:4-dinitrophenylhydrazone. Neither *cyclo*hexanol nor *cyclo*hexanone was found in the expired air and therefore further details of their determination are omitted.

Determination of urinary metabolites

cyclo*Hexanol.* Carrier *cyclo*hexanol (1 g.) was added to a volume of urine (usually 3–5 ml.) sufficient to contain about 1 μc of radioactivity. Half a volume of 10N-HCl was added and the mixture refluxed for 3 hr. The cooled mixture was continuously extracted with ether for 3 hr. After washing the extract with saturated $NaHCO_3$ soln, the ether was evaporated and the residual *cyclo*hexanol dried with anhydrous Na_2SO_4. The 3:5-dinitrobenzoate was prepared and washed successively with 2N-HCl, saturated Na_2CO_3 soln. and water until it was neutral. It was recrystallized from ethanol (charcoal) to constant activity (m.p. 110°). Free *cyclo*hexanol in the urine was determined in a similar manner but the hydrolysis with HCl was omitted.

cyclo*Hexanone.* This ketone (0·3 g.) was substituted for the *cyclo*hexanol described in the preceding paragraph and the hydrolysis was carried out similarly. The hydrolysate was steam-distilled and the *cyclo*hexanone in the distillate was extracted with ether. The dried (anhydrous Na_2SO_4) extract was evaporated and the recovered ketone dissolved in ethanol and added to a boiling solution of 2:4-dinitrophenylhydrazine [10%, w/v, in H_2SO_4–ethanol (1:5, v/v)]. After boiling for 1 min., the solution was cooled and the 2:4-dinitrophenylhydrazone of *cyclo*hexanone which separated was recrystallized to constant activity (m.p. 157–158°). Free *cyclo*hexanone was determined by the same procedure but the acid hydrolysis was omitted. No free *cyclo*hexanone was found and the ketone found after hydrolysis is probably an artifact (see below).

cis- *and* trans-cyclo*Hexane-1:2-diol.* Carrier amounts (0·25 g.) of each diol were added to urine and the hydrolysis was carried out as for *cyclo*hexanol. Extraction of the diols was made when the hydrolysate had been adjusted to pH 8 with NaOH. The ether was evaporated and the residue was then washed with ether to remove *cyclo*hexanol. The residue, dissolved in pyridine, was then treated with *p*-nitrobenzoyl chloride at 100° for 10 min. The solid residue was stirred with water and extracted successively with 2N-HCl, saturated Na_2CO_3 soln. and finally with water until neutral and free from pyridine. The di-*p*-nitrobenzoates of the diols (m.p. *trans*-, 130°; *cis*, 149°; see Elliott, 1958) were then

repeatedly recrystallized from ethanol to constant activity. The free diols were determined in the same way but the hydrolysis was omitted.

Some determinations of *cyclo*hexanol, *cyclo*hexanone, *cyclo*hexane-1:2-diols and *cyclo*pentanealdehyde were carried out after hydrolysis of the urine with β-glucuronidase. Generally, the urine (8–10 ml.) was adjusted to pH 4·6 and then incubated at 30° for 24 hr. with 10 mg. of an ox-liver β-glucuronidase preparation (3000 Fishman units: L. Light and Co.). The necessary carrier was then added to the mixture, which was then incubated for a further 24 hr., and the material to be determined was then isolated and counted as described above.

trans-cyclo*Hexane*-1:3-*diol*. This was determined similarly after the addition of 250 mg. of carrier, except that it was isolated finally as the dibenzoate [m.p. 124° after crystallization from methanol, light petroleum (b.p. 60°), acetic acid and finally ethanol].

trans-cyclo*Hexane*-1:4-*diol*. The above described procedure could not be successfully applied to the 1:4-diol owing to its low solubility in ether. The 1:4-diol (150 mg.) was added to 3–4 ml. of urine and the mixture (adjusted to pH 4·5) incubated for 24 hr. at 37° with a β-glucuronidase extract from *Helix pomatia*. The incubation mixture was evaporated to dryness at not more than 40° and the residue extracted repeatedly with ethanol. The ethanol was removed and the residue dissolved in pyridine and benzoylated with benzoyl chloride. The dibenzoate of *trans-cyclo*hexane-1:4-diol was recrystallized from ethanol until it had no activity (m.p. 150°).

cyclo*Hexane*-1:2-*dione*. The ketone (0·25 g.) was dissolved in 1·7 ml. of urine and hydroxylamine hydrochloride (0·44 g.) added, the solution being kept at 0°. A 50% (w/v) solution of KOH (0·7 ml.) was added drop by drop at 0°, and after keeping at 0° for 1 hr. the *cyclo*hexane-dione dioxime was collected and recrystallized from water (m.p. 187–188°). It had no activity.

cyclo*Hexene oxide*. The oxide (0·4 ml.) was added to urine (8 ml.) adjusted to pH 7. After 24 hr. the mixture was centrifuged. The supernatant oxide was separated and converted into *trans-cyclo*hexane-1:2-diol with 2N-H_2SO_4 (8 ml.). The diol was extracted with ethyl acetate, the extract dried with anhydrous Na_2SO_4 and the solvent evaporated. The diol was then counted as its di-*p*-nitrobenzoate as already described. No activity was found.

cyclo*Pentanealdehyde*. This highly volatile compound was lost during acid hydrolysis. A mixture of urine (8 ml.) and concentrated HCl (4 ml.) was heated in a sealed tube at 115° for 3 hr. After cooling, the tube was opened and its contents were neutralized with solid $NaHCO_3$. An excess of acetic acid (about 1 ml.) was added, followed by *trans*-2-amino*cyclo*hexanol (230 mg.). The mixture was cooled to 0° and sodium nitrite (690 mg. in 2 ml. of water) added to convert the amino compound into *cyclo*pentanealdehyde (McCasland, 1951). Excess of nitrous acid was removed with aq. 1% ammonium sulphamate soln. and the mixture steam-distilled. The distillate (10 ml.) was treated with an excess of a saturated solution of 2:4-dinitrophenylhydrazine in ethanol. The *cyclo*pentanealdehyde 2:4-dinitrophenylhydrazone which separated was recrystallized from ethanol to constant specific activity (m.p. and mixed m.p. 158°). For enzymic hydrolysis, the urine (8 ml.) was brought to pH 4·6 and incubated in a sealed tube with 1 ml. of a β-glucuronidase preparation from the giant African snail (*Achatina fulica*) at 30° for 24 hr. The carrier *cyclo*pentanealdehyde was prepared in the mixture from *trans*-2-amino-*cyclo*hexanol and counted as described above.

Succinic acid. This was determined as described by Parke & Williams (1953) except that the benzhydrol derivative of succinic acid was not prepared.

Oxalic acid. This was also determined according to Parke & Williams, but acetic acid was used instead of HCl for acidifying the urine.

Adipic acid. A solution of adipic acid (0·25 g.) in 2 ml. of 2N-Na_2CO_3 was added to urine (3–5 ml.) which had been previously acidified with HNO_3 and titrated with $AgNO_3$ soln. to remove chlorides. The mixture was treated with 3 ml. of aq. 10% (w/v) $AgNO_3$ soln. and the silver adipate was precipitated by dropwise addition of aq. 2N-NH_3 soln. The precipitate was filtered off and twice reprecipitated from solution in 2N-HNO_3 by addition of ammonia. The adipic acid was then removed from an acid solution by extraction with ethyl acetate and was recrystallized from ethanol until its activity disappeared (m.p. 152°).

Maleic acid. The acid (250 mg.) was added to 8 ml. of urine previously incubated for 24 hr. at 30° with 10 mg. of an ox-liver β-glucuronidase preparation (3000 Fishman units). After 3 hr. an excess of aq. 10% (w/v) $BaCl_2$ soln. was added to the solution and the barium maleate precipitated by dropwise addition of aq. 2N-NH_3 soln. The precipitate was dissolved in 2N-HCl and the maleic acid extracted from the solution with ethyl acetate. Removal of the ethyl acetate gave maleic acid, m.p. 135°, which showed no radioactivity.

Malonic acid. The acid (0·25 g.) dissolved in 2 ml. of 2N-NaOH was added to a volume of urine containing approximately 1 μc of radioactivity and the mixture adjusted to pH 2 by the addition of 2N-HCl. The urine was then continuously extracted with ether for 16 hr. The crystalline precipitate obtained was dissolved in ethanol, and the solution, after decolorizing with charcoal, was evaporated to dryness. Barium hydroxide solution (0·25N) was added to the residue, and the precipitated barium salt was dissolved in 2N-HCl, reprecipitated by the addition of barium hydroxide (0·25N) and digested with ethanol. Malonic acid (m.p. 133°) was extracted with ether in a continuous extraction from the barium salt after acidification with HCl, and it was finally recrystallized from ethanol until its activity disappeared.

Isolation of metabolites

From cyclo*hexane*. The 24 hr. urine of four rabbits, which had received collectively 4·8 g. of *cyclo*hexane, was neutral, did not reduce Benedict's reagent and gave an intense naphtharesorcinol reaction. Chromatography of the urine in solvents B and C (see Table 1) showed the presence of two glucuronides (one of R_F 0·55 in B, and 0·59 in C; the other, R_F 0·45 in B and 0·42 in C). The first corresponded to *cyclo*hexyl glucuronide and the second to *cyclo*hexane-1:2-diol glucuronide. One of the glucuronides was isolated through the basic lead acetate fraction of the urine (Kamil, Smith & Williams, 1951) as a gum, which on methylating with diazomethane and acetylating with pyridine and acetic anhydride yielded 2·2 g. of a crude solid. On repeated recrystallization from aq. 70% ethanol and finally ethanol, *methyl* (cyclo*hexyl tri*-O-*acetyl*-β-D-*glucosid*)*uronate* (0·4 g.) was obtained as white needles, m.p. 139° and $[\alpha]_D^{20}$ – 35·7° in

$CHCl_3$ (*c*, 1) (Found: C, 54·7; H, 6·9; $C_{19}H_{28}O_{10}$ requires C, 54·8; H, 6·8%). (Optical rotations are given to ±1°.)

From cyclo*hexanol*. Paper chromatography of the urine showed the same glucuronide spots as in *cyclo*hexane urine (see above). The same methyl (*cyclo*hexyl tri-*O*-acetyl-β-D-glucosid)uronate was isolated in a similar manner (see above) from the urine of six rabbits which had received 1 g. each of *cyclo*hexanol. The yield of crude ester was 6·5 g., which on repeated recrystallization had m.p. and mixed m.p. 139° and $[\alpha]_D^{20}$ −35·6° in $CHCl_3$ (Found: C, 55·0; H, 7·0; CH_3CO, 31·2. $C_{19}H_{28}O_{10}$ requires C, 54·8; H, 6·8; CH_3CO, 31%). The ester (0·5 g.) was refluxed for 3 hr. with N-HCl (5 ml.). After cooling, the solution was extracted with ether. The extract was dried (anhydr. Na_2SO_4) and evaporated. The residue was esterified with 3:5-dinitrobenzoyl chloride and the resulting *cyclo*hexyl 3:5-dinitrobenzoate had m.p. and mixed m.p. 110° after repeated recrystallization from ethanol.

From cyclo*hexanone*. Four rabbits were each given 1·5 g. of *cyclo*hexanone. The urine, which was collected for 18 hr., was non-reducing and was free from ketones, but the naphtharesorcinol reaction was strongly positive. The glucuronide was isolated as above (yield 6·1 g. crude) and from this pure methyl (*cyclo*hexyl tri-*O*-acetylglucosid)-uronate was obtained, m.p. and mixed m.p. 139° and $[\alpha]_D^{26}$ −36·0° in $CHCl_3$.

From cyclo*hex*-1-*en*-1-*yl acetate*. Free *cyclo*hex-1-en-1-ol is unstable and readily isomerizes to *cyclo*hexanone. The acetate (6 g. to four rabbits) was therefore fed. The urine (after 18 hr.) was non-reducing and contained a glucuronide. This proved to be that of *cyclo*hexanol, for 4·3 g. of methyl (*cyclo*hexyl tri-*O*-acetylglucosid)uronate (m.p. 139° after crystallization and $[\alpha]_D^{26}$ −35·2° in $CHCl_3$) was isolated.

From (±)-trans-cyclo*hexan*-1:2-*diol*. A total of 6 g. of the diol was fed to four rabbits. The 18 hr. urine was non-reducing, contained no ketones and gave a strong naphtharesorcinol reaction. Paper chromatography of the urine with solvents B and C (Table 1) revealed one spot reacting with naphtharesorcinol, with R_F 0·45 in B and 0·42 in C. The glucuronide gum (5·5 g.) was prepared through basic lead acetate precipitation and part of it slowly crystallized to give a glucuronide, m.p. 135–140°, which could not be purified readily. The main bulk was methylated and acetylated to give 7·9 g. of crude ester. Repeated recrystallizations from ethanol yielded white needles of *methyl* (2-*acetoxy*cyclo*hexyl tri*-O-*acetyl*-β-D-*glucosid)uronate*, m.p. 165·5–166°; $[\alpha]_D^{20}$ −31·9° in $CHCl_3$ (*c*, 1) (Found: C, 53·9; H, 6·3; CH_3CO, 36·9. $C_{21}H_{30}O_{12}$ requires C, 53·2; H, 6·4; CH_3CO, 36·3%). Hydrolysis of this ester (0·35 g.) with N-HCl, extraction with ether and esterification of the residue with *p*-nitrobenzoyl chloride as described above for the *cyclo*hexyl glucuronide yielded the di-*p*-nitrobenzoate of (±)-*trans-cyclo*hexane-1:2-diol, m.p. and mixed m.p. 148·5° after recrystallization.

*cis-cyclo*Hexane-1:2-diol was also fed to rabbits (1 g. each), but although a glucuronide was formed, it was isolated as a gum which was not successfully crystallized, nor could a crystalline derivative be prepared from it.

RESULTS AND DISCUSSION

Glucuronide formation. Quantitative results for the glucuronide output of rabbits receiving oral doses of *cyclo*hexane and its derivatives are shown in Table 2. At a dose of 460 mg./kg., about 30% of *cyclo*hexane is excreted as glucuronides, of which there are two. The main one is *cyclo*hexyl glucuronide, which was isolated, and the minor one is *trans-cyclo*hexane-1:2-diol glucuronide. The latter was shown to be present by paper chromatography, and by the detection of *trans-cyclo*hexane-1:2-diol by isotope dilution after hydrolysis with β-glucuronidase of the urine obtained after dosing with *cyclo*[^{14}C]hexane.

*cyclo*Hexanol is highly conjugated with glucuronic acid (Table 2) and gives rise to the same two glucuronides as *cyclo*hexane. *cyclo*Hexanone also gives rise to considerable amounts of conjugated glucuronic acid which was proved, by isolation, to be mainly *cyclo*hexylglucuronide. *cyclo*Hexanone is therefore reduced to *cyclo*hexanol *in vivo*. *cyclo*Hex-1-enyl acetate is metabolized also to *cyclo*hexyl glucuronide. This suggests that it is hydrolysed to *cyclo*hex-1-enol, which is known to rearrange rapidly to *cyclo*hexanone; the latter is then reduced and conjugated as shown in Fig. 1.

Glucuronide formation is also the main fate of the *cis*- and *trans-cyclo*hexane-1:2-diol. The *cis* isomer seems to be more highly conjugated than the *trans*, but only in the latter was a crystalline derivative of the glucuronide, which proved to be a monoglucuronide, isolated.

Metabolism of cyclo*hexane*. The quantitative aspects of the metabolism of *cyclo*hexane are shown in Tables 3 and 4. Most of the experiments were carried out at a dose of 300–400 mg./kg. In one experiment, however, the dose was a thousand times smaller, namely 0·3 mg./kg. Table 3 shows the results for metabolites eliminated in the ex-

Table 2. *Conjugation of* cyclo*hexane and its derivatives by the rabbit*

Figures for conjugation are the means for three animals, the ranges being in parentheses, except for *cyclo*hexanol, where two animals were used.

Compound administered	Dose† (mg./kg.)	Percentage of dose excreted as glucuronide
*cyclo*Hexane*	460	30 (27–37)
*cyclo*Hexanol‡	260	65 (61, 68)[2]
*cyclo*Hexanone	248	66 (51–86)
*cyclo*Hex-1-enyl acetate	350	39 (37–42)
(±)-*trans-cyclo*Hexane-1:2-diol	280	49 (42–56)
*cis-cyclo*Hexane-1:2-diol	280	75 (63–89)

* Ethereal sulphate and mercapturic acid outputs were measured for *cyclo*hexane, but they were not significantly different from normal.

† About 5·5 m-mole/kg. for *cyclo*hexane, and 2·5 m-mole/kg. for the other compounds.

‡ *cyclo*[^{14}C]Hexanol of low activity, and prepared biosynthetically from *cyclo*[^{14}C]hexane. In the two animals used the total radioactivity of the urine was 68 and 75% of the dose; these figures are slightly higher than the glucuronide figures of 61 and 68% respectively.

pired air. When the dose is 300–400 mg./kg., about 35–45 % of the dose is eliminated in the expired air, some 10 % of the dose as carbon dioxide and 25–35 % as unchanged *cyclo*hexane (b.p. 81°). When the dose is very small (Expt. 4) no unchanged *cyclo*hexane is eliminated, but about 5 % is converted into carbon dioxide, the rest being mainly excreted in the urine as metabolites. In Expts. 2–4, quoted in Table 3, the radioactivity accounted for was almost quantitative and amounted to about 95 % of the dose.

Table 4 summarizes our isotope-dilution experiments on the identification of the metabolites of *cyclo*hexane. According to this table, the main metabolite is *cyclo*hexanol, which accounts for 30–40 % of the high dose and 60 % of the low dose. The next main metabolite is (±)*trans-cyclo*hexane-1:2-diol, which accounts for 5–8 % of the high dose and nearly 17 % of the low dose. These two compounds, conjugated with glucuronic acid, appear to be the only urinary metabolites of *cyclo*hexane in the rabbit. However, there are quoted in this table small positive values for *cis-cyclo*hexane-1:2-diol, *cyclo*hexanone and phenol. Furthermore, in Table 5 a small positive value is also recorded for *cyclo*pentanealdehyde. We have evidence, however,

Table 3. *Metabolites of* cyclo*hexane in the expired air*

[^{14}C]*cyclo*Hexane was administered orally to rabbits and the expired air was collected for 2 days.

Expt. no.	2	3	4
Dose of *cyclo*hexane (mg./kg.)	390	360	0·3†
Dose of ^{14}C (μc/animal)	108	185	50
Metabolites (% of dose)			
Carbon dioxide	10·1	8·5	5·5
*cyclo*Hexane	24·5	38·0	0
*cyclo*Hexanol	0	0	0
*cyclo*Hexanone	0	0	0
Total in expired air	34·6	46·5	5·5
(Urine, faeces and tissues*	58·7	49·8	89·2)
(Total accounted for	93·3	96·3	94·7)

* See Table 4.

† This small dose consisted of 0·7 mg. of *cyclo*hexane dissolved in 0·8 ml. of arachis oil.

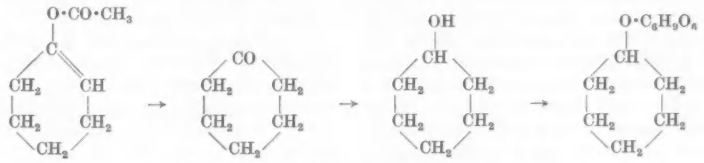


Fig. 1. Reduction and conjugation of *cyclo*hexanone.

Table 4. *Urinary metabolites of* cyclo*hexane*

Metabolites found are expressed in % of dose.

Expt. no.	1	2	3	4	5
Dose of *cyclo*hexane (mg./kg.)	330	390	360	0·3	100‡
Dose of ^{14}C (μc/animal)	18·5	108	185	50	14
Duration of experiment (days)	5	6	3	4	2
Metabolites examined‖					
*cyclo*Hexanol	—	41 (1·0)*	34·4	61·0	48·0
(±)-*trans-cyclo*Hexane-1:2-diol	4·5	8·5 (0·07)*	4·7	16·7	—
*cis-cyclo*Hexane-1:2-diol§	0·4	0·9	1·1	3·5	—
*cyclo*Hexanone§	1·5	3·6 (0)*	5·4 (0)*	4·5 (0)*	—
*cyclo*Hexane-1:2-dione	—	0	0	0	—
*trans-cyclo*Hexane-1:3-diol	—	0	0	0	—
*trans-cyclo*Hexane-1:4-diol	—	0	0	0	—
*cyclo*Hexene oxide	—	—	—	—	0
Adipic acid	0	0	0	0	—
Succinic acid	—	0	0	0	—
Malonic acid	—	0	0	0	—
Oxalic acid	0	0	0	0	—
(Phenol)	—	(0·6)†	(0·5)†	(1·1)†	(<0·02)
Sum of urinary metabolites	—	54·6	46·2	86·8	48·0
Radioactivity of urine	33·0	56·0	41·0	98·0	59
Radioactivity of faeces	—	0·2	0·1	—	—
Radioactivity of tissues	—	2·6	2·4	—	—

* These figures in parentheses are for the free compound, that is before acid hydrolysis.

† This phenol is derived from the [^{14}C]benzene present in the *cyclo*hexane.

‡ Benzene-free *cyclo*hexane. § Artifacts: see Table 5 and text. ‖ Determined by isotope-dilution.

which suggests that three of these compounds are artifacts of the acid-hydrolysis procedures used in the experiments. The appearance of phenol in amounts of 0·5–1·1% of the dose in Expts. 2–4 and 7 is due to the presence of small amounts of benzene in the *cyclo*[^{14}C]hexane supplied to us. When a specially purified sample of *cyclo*[^{14}C]-hexane which had been freed of [^{14}C]benzene by gas chromatography was fed to rabbits, no phenol was found in the urine (see Expt. 5, Table 4). Thus it was proved that *cyclo*hexane was not aromatized and converted into phenol *in vivo*.

*cis-cyclo*Hexane-1:2-diol, *cyclo*hexanone and *cyclo*-pentanealdehyde appear to be artifacts. When the urine is hydrolysed with β-glucuronidase instead of hydrochloric acid, these compounds were not found in the hydrolysate (see Table 5). However, the origin of these artifacts is not entirely clear, but they could arise from *trans-cyclo*hexane-1:2-diol glucuronide. It was found that when either (±)-*trans-cyclo*hexane-1:2-diol or its non-crystalline glucuronide was heated with 5N-hydrochloric acid, small amounts of *cyclo*pentanealdehyde could be isolated (the 2:4-dinitrophenylhydrazone, m.p. 155–156°, not depressing the m.p. of the authentic compound, m.p. 158–159°, but depressing the m.p. of *cyclo*hexanone 2:4-dinitrophenylhydrazone, m.p. 157–158°). On heating *cis-cyclo*hexane-1:2-diol in the same way, small amounts of *cyclo*-hexanone were isolated (2:4-dinitrophenylhydrazone, m.p. and mixed m.p. 158°, but depressing the m.p. of *cyclo*pentanealdehyde 2:4-dinitrophenyl-hydrazone). When the non-crystalline *cis-cyclo*-hexane-1:2-diol glucuronide was heated with hydrochloric acid, the 2:4-dinitrophenylhydrazone (m.p. 125–127°) of an aldehyde or a ketone was isolated but it depressed the m.p. of both the 2:4-dinitrophenylhydrazone of *cyclo*pentanealdehyde and of *cyclo*hexanone; we suspect that this compound was a mixture of the two dinitrophenyl-hydrazones, but we were unable to separate them. Under certain conditions derivatives of the *trans*-diol can be converted into the *cis*-diol, and there is a considerable literature on this point (see, for example, Clarke & Owen, 1949; Winstein, Hess & Buckles, 1942; Fujita & Nomura, 1942). We suggest, tentatively, that the small amount of *cis-cyclo*hexane-1:2-diol, *cyclo*hexanone and *cyclo*-pentanealdehyde found in the urine after acid hydrolysis are derived from *trans-cyclo*hexane-1:2-diol glucuronide thus:

trans-diol glucuronide → *cis*-diol → *cyclo*hexanone
trans-diol glucuronide → *cyclo*pentanealdehyde

Other *cyclo*hexane derivatives tested for were *cyclo*hexane-1:2-dione, *trans-cyclo*hexane-1:3- and -1:4-diol, and *cyclo*hexene oxide, but they were not found.

Since *cyclo*hexane is converted into carbon dioxide, possible intermediates are the dicarboxylic acids adipic, succinic, maleic, malonic and oxalic, but these were not detected in the urine by isotope dilution.

Table 6 shows the ratio of *cyclo*hexanol to *trans-cyclo*hexane-1:2-diol in the urine of rabbits at

Table 5. *Urinary metabolites of* cyclo*hexane determined after acid and enzymic hydrolysis of urine*

Expt. no.	6	7
Dose of *cyclo*hexane (mg./kg.)	272	221
Dose of ^{14}C (μc/animal)	20·1	91·5
Duration of experiment (hr.)	48	48
	Acid hydrolysis	Enzymic hydrolysis
Metabolites (% of dose)*		
*cyclo*Hexanol	39·5	41·1
(±)-*trans-cyclo*Hexane-1:2-diol	2·95	3·1
*cis-cyclo*Hexane-1:2-diol	1·8	0
*cyclo*Hexanone	1·4	0
*cyclo*Hex-2-enol	—	0
*cyclo*Pentanealdehyde	1·3	0
Adipic acid	—	0
Maleic acid	—	0
(Phenol)	—	(0·6)
Sum of metabolites	47·0	44·8
Radioactivity of urine	49·3	51·0

* Determined by isotope-dilution.

Table 6. *Relative excretion of* cyclo*hexanol and* trans-cyclo*hexane-1:2-diol at various times after dosing with* cyclo*hexane*

Expt. 3
(dose of *cyclo*hexane, 360 mg./kg.)

Time of collection of urine after dosing (hr.)	Ratio mono-ol/diol*
0–18	18·0
18–42	7·0
42–48	6·8

Ratio for total excretion is about 7.

Expt. 4
(dose of *cyclo*hexane, 0·3 mg./kg.)

Time of collection of urine after dosing (hr.)	Ratio mono-ol/diol*
0–1·5	9·0
1·5–6	5·3
6–24	4·6
24–48	3·0

Ratio for total excretion is about 4.

* This is the ratio of the amount of *cyclo*hexanol to *trans-cyclo*hexane-1:2-diol excreted in that time.

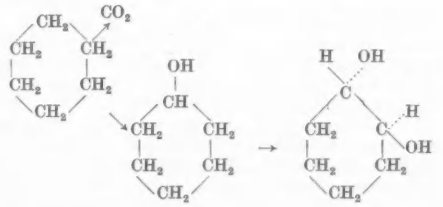
Table 7. *Some metabolites of* [^{14}C]cyclo*hexanol*

Expt. no.	1	2
Dose (mg./kg.)	270	260
Dose of radioactivity (μc/animal)	1	1
	Percentage of dose*	
*cyclo*Hexanol	58·0	64·5
*trans-cyclo*Hexane-1:2-diol	7·3	5·5
*cyclo*Hexanone†	5·3	6·1
Sum of metabolites	65·3‡	70·0‡
Total radioactivity of urine	—§	75·0

* These figures were obtained after acid hydrolysis of the urine, which had been collected for 2 days after dosing.
† *cyclo*Hexanone is probably an artifact, see text.
‡ Excluding *cyclo*hexanone.
§ Urine lost.

various times after dosing. It is to be noted that, in both experiments, the ratio falls with time. This suggests that the primary metabolite is *cyclo*hexanol and that the diol is being formed from the mono-ol. The diol is, in fact, a metabolite of *cyclo*hexanol, as shown in Table 7. In the two experiments quoted, 58 and 64 % of the dose was excreted as conjugated *cyclo*hexanol, and 7·3 and 5·5 % as conjugated *trans-cyclo*hexane-1:2-diol. The sum of these two metabolites, 65 and 70 %, is in agreement with the corresponding glucuronide figures, 61 and 68 % quoted in Table 2.

Our results suggest that the gross outlines of the metabolism of *cyclo*hexane are as follows:



At present there is no evidence to show how carbon dioxide is formed or in which metabolite ring-opening occurs.

SUMMARY

1. When *cyclo*[^{14}C]hexane is administered to rabbits in doses of about 350–400 mg./kg., about 40 % of the ^{14}C appears in the expired air and about 50 % in the urine in 2 days after dosing. With a dose of 0·3 mg./kg., only about 5 % of the dose of ^{14}C is found in the expired air and 90 % in the urine.

2. With the large dose, the ^{14}C-labelled compounds found in the expired air were carbon dioxide (about 10 % of the dose) and unchanged *cyclo*hexane (about 30 %), whereas with the small dose only labelled carbon dioxide (5·5 %) was found.

3. The urinary metabolites of *cyclo*hexane were *cyclo*hexanol and *trans-cyclo*hexane-1:2-diol, excreted as glucuronides. *cyclo*Hexyl glucuronide was isolated and characterized. With the larger doses the *cyclo*hexanol accounted for 35–50 % and the diol for 3–8 % of the dose, whereas the values for the small dose were *cyclo*hexanol, 61 %, and the diol, 16·7 %. The ratio, *cyclo*hexanol/*cyclo*hexane-diol, was about the same for the large and small doses.

4. *cis-cyclo*Hexane-1:2-diol, *cyclo*hexanone, *cyclo*hexane-1:2-dione, *trans-cyclo*hexane-1:3- and -1:4-diol, *cyclo*hexene oxide, *cyclo*hex-2-en-1-ol, and adipic acid, succinic acid, maleic acid, malonic acid and oxalic acid, and phenol, do not appear to be urinary metabolites of *cyclo*hexane.

5. *cyclo*Hexanol is highly conjugated (65 % of the dose) with glucuronic acid in the rabbit. The main metabolite is *cyclo*hexyl glucuronide. About 6 % of the dose is excreted as conjugated *trans-cyclo*hexane-1:2-diol.

6. *cyclo*Hexanone is reduced in rabbits and excreted mainly as *cyclo*hexyl glucuronide. This glucuronide is also the main metabolite of *cyclo*hex-1-en-1-ol, which was fed as its acetate.

7. Both *cis-* and (±)*trans-cyclo*hexane-1:2-diol are excreted by rabbits, highly conjugated with glucuronic acid. The monoglucuronide of the *trans*-diol was isolated and characterized.

One of us (T.H.E.) participated in this work during sabbatical leave from the University of Malaya, Singapore.

REFERENCES

Arnold. R. T. (1952). *Org. Synth.* **32**, 35.
Baldwin, B. C., Robinson, D. & Williams, R. T. (1959). *Biochem. J.* **71**, 638.
Bartlett, P. D. & Woods, G. F. (1940). *J. Amer. chem. Soc.* **62**, 2933.
Bernhard, K. (1937). *Hoppe-Seyl. Z.* **248**, 256.
Browning, E. (1953). *Toxicity of Industrial Solvents*, p. 94. London: H.M. Stationery Office.
Clarke, M. F. & Owen, L. N. (1949). *J. chem. Soc.* p. 315.
Clarke, M. F. & Owen, L. N. (1950). *J. chem. Soc.* p. 2103.
Elliott, T. H. (1958). *J. chem. Soc.* p. 3752.
Fabre, R., Truhaut, R. & Peron, M. (1952). *Arch. Mal. prof.* **13**, 437.
Filippi, E. (1914). *Arch. Farmacol. sper.* **18**, 178.
Fujita, S. & Nomura, K. (1942). *J. chem. Soc. Japan*, **63**, 510.
Gilman, H. & Blatt, A. H. (1941). *Org. Synth. Coll.* **1**, 185.
Henbest, H. B. & Wilson, R. A. L. (1957). *J. chem. Soc.* p. 1958.
Horning, E. C. (1955). *Org. Synth. Coll.* **3**, 217.
Imelik, B. (1948). *C.R. Soc. Biol., Paris*, **226**, 2082.
Kamil, I. A., Smith, J. N. & Williams, R. T. (1951). *Biochem. J.* **50**, 235.
McCasland, G. E. (1951). *J. Amer. chem. Soc.* **73**, 2293.

Machinskaya, I. V. (1952). *J. gen. Chem., Moscow*, **22**, 1159. Cited in *Chem. Abstr.* **47**, 6357.
Mead, J. A. R., Smith, J. N. & Williams, R. T. (1958). *Biochem. J.* **68**, 61.
Parke, D. V. & Williams, R. T. (1950). *Biochem. J.* **46**, 236.
Parke, D. V. & Williams, R. T. (1953). *Biochem. J.* **54**, 231.
Torboli, A. (1937). *Boll. Soc. ital. Biol. sper.* **12**, 368.
Treon, J. F., Crutchfield, W. E. jun. & Kitzmiller, K. V. (1943). *J. industr. Hyg.* **25**, 199.
Van Slyke, D. D., Plazin, J. & Weisiger, J. R. (1951). *J. biol. Chem.* **191**, 299.
Vogel, A. I. (1956). *Practical Organic Chemistry*, 3rd ed. p. 895. London: Longmans, Green and Co.
Weitzel, G. (1950). *Hoppe-Seyl. Z.* **285**, 58.
Wilson, N. H. B. & Read, J. (1935). *J. chem. Soc.* p. 1268
Winstein, S., Hess, H. V. & Buckles, R. E. (1942). *J. Amer. chem. Soc.* **64**, 2796.

Haemorrhage and Tissue Electrolytes

By E. M. WIDDOWSON and D. A. T. SOUTHGATE
Medical Research Council Department of Experimental Medicine, University of Cambridge

(*Received* 13 *June* 1958)

A survey of the literature reveals considerable variability in the concentration of chloride in skeletal muscle. The variability has been both from one investigator to another and from one animal to another. Darrow, Harrison & Taffel (1939), for example, analysed the muscle of four normal dogs and found the chloride concentration to vary from 18·9 to 22·8 m-equiv./kg., and Nichols, Nichols, Weil & Wallace (1953) obtained similar figures. Kerpel-Fronius (1937), however, found 12·7 m-equiv./kg. Most of the published figures for the skeletal muscle of rats, rabbits and cats have been of the order of 12–14 m-equiv./kg. (Kerpel-Fronius, 1937; Darrow *et al.* 1939; Hines & Knowlton, 1939; Manery & Hastings, 1939; Lowry, Hastings, Hull & Brown, 1942). The concentration of chloride in human muscle has generally been found to be as high as or higher than those given by Darrow *et al.* (1939) for dogs. Kerpel-Fronius (1937) found 16·9 and 18·6 m-equiv./kg. in samples from two men, Talso, Spafford & Blaw (1953) 19·1, Wilson (1955) an average of 25·6, with a range from 19·3 to 38·3, Mudge & Vislocky (1949) 27·4, 28·1 and 29·7 and Barnes, Gordon & Cope (1957) an average of 23·1 with a range from 13·9 to 39·1 m-equiv./kg. There has not been the same variability in the concentration of chloride in other tissues, but since chloride is generally considered to be outside the cells, and to give a fair measure of the extracellular-fluid volume of an adult muscle we began to consider why the chloride in muscle was so variable. Even taking into consideration the facts that some authors express their results on a fat-free basis, whereas others do not, and that some but not others rinse their samples and blot them to remove superficial blood, the variation still remained very great, and it was clear that there was some other cause of the variability. The fact that bleeding the animal might have something to do with it was brought home to us when we compared the chloride concentrations in the muscles of pigs which had been killed and bled at the slaughter-house with those from pigs which had not been bled. The average for the former group was 13 m-equiv./kg., that for the latter 22 m-equiv./kg. It was decided to look into this matter further and to investigate the effect of bleeding on the chemical composition of skeletal muscle of the rat, the fowl, the pig and man. Since, moreover, we wished to know whether chloride could be used as a measure of the extracellular fluid in young animals it was decided to try the effect of bleeding and at the same time to compare the chloride and inulin spaces in the tissues of the newborn pig.

EXPERIMENTAL

Animals and experiments

Rats. Five adult black-and-white hooded rats weighing about 300 g. were anaesthetized with ether and as much as possible of the adductor and quadriceps muscles were taken from the right thigh. Then each animal was killed by bleeding from the abdominal aorta, and when it was dead the same muscles were taken from the other thigh. The amount of blood that could be removed before the animal died was not more than 5–8 ml.

A muscle taken from a living body is quite different in appearance from one taken from the body after it is dead. It is bright pink, with blood oozing from the small capillaries, whereas there is no visible blood in an autopsy specimen. It was thought that this in itself might affect the composition quite apart from any differences due to the removal of blood from the animal. In order to control this point, five other similar rats were anaesthetized as before, muscle was removed from the right thighs, the animals were killed with an overdose of ether and muscle was taken from the other side.

Cockerels. A healthy Rhode Island cockerel aged 26 weeks, weighing 3·5 kg., was killed by decapitation and the bird was exsanguinated by hanging it head downwards.

A second similar bird was killed by an overdose of ether and was not bled. Samples of pectoral muscle were taken from each animal.

Pigs: effects of bleeding. The pigs came from the Department's colony. They were all healthy at death. They were descendants of a pure-bred Large White × Essex cross subsequently mated with pure-bred Large White boars.

Muscle was removed from the thighs of four pigs weighing about 200 kg. which had been killed at the slaughterhouse and had had their throats cut immediately after they had been electrocuted. The corresponding muscle was also taken from two animals of the same weight and breed which had not been bled.

In one newborn pig, weighing 1·5 kg., muscle was removed from one thigh under pentobarbitone sodium (Nembutal) anaesthesia. Then 20 ml. of blood was taken from the heart and the animal killed by injecting air into the heart. Muscle was then taken from the other thigh and the two samples were analysed at the same time. The liver and heart were also taken for analysis. This piglet was a litter mate of the one to which inulin was given (see below), and served as its control.

Pigs: comparisons of inulin and chloride. Two piglets born in the evening were left with the sow for 10 hr., then removed to metabolism cages and given no further food. A volume (5 ml.) of 10% inulin solution was administered by cardiac puncture to one piglet, and any urine passed subsequently was collected. Two hours later 1·5 ml. of the 10% inulin solution was given subcutaneously, and 2 ml. was given after another 3 hr. The piglet was anaesthetized with pentobarbitone sodium 8 hr. after the first injection: 20 ml. of blood was removed from the heart and the serum separated. The animal was then killed by injecting air into the heart, and thigh muscle, liver and heart were removed for analysis. The control piglet, which received no inulin, was treated as described above, and its blood serum was also separated.

The serum, skeletal muscle, heart and liver of the two animals were analysed for inulin in duplicate, side by side, and the value obtained for the control animal was deducted from the one obtained for the experimental animal and the difference taken to be the concentration of 'true inulin' in the organ.

Man. Muscle from four men who had died as a result of street accidents were available for this comparison. Two of the men had sustained severe haemorrhage as a result of the accident and two had not.

Sampling and chemical methods

The muscle was freed from fat and connective tissue as far as possible and cut finely with scissors. The livers of the two newborn piglets were homogenized without added water in a Waring Blendor. The whole of the piglets' hearts were also cut up by hand. Weighed samples of the fresh material were dried to constant weight in silica crucibles at 100°, then ashed in a muffle furnace at 450°; the ash was extracted, first with conc. HCl and subsequently with 0·5N-acid as described by McCance, Widdowson & Shackleton (1936), and the extract used for the determination of sodium, potassium and phosphorus.

At first some sodium and potassium determinations were made in a Beckman flame photometer on the acid extracts of the ash, but it was soon realized that the results obtained in this way were only 60–70% of the values obtained by chemical methods. Crismon (1948) and Bernstein (1952) also observed a reduction of emissions when working with acid extracts of tissue ashes, but they found complete agreement between chemical and flame-photometric methods after removal of phosphates. In the present investigation the Beckman flame photometer was used for the determination of sodium, but a measured amount of the acid extract was first evaporated to dryness and heated for 16–18 hr. at 100° to remove all traces of acid, and taken up with water, shaken with solid $Ca(OH)_2$ to remove phosphates and filtered. Results obtained in this way agreed well with those found by the more laborious chemical method with zinc uranyl acetate as precipitant. Potassium was determined chemically with sodium cobaltinitrite as described by McCance & Shipp (1933). Phosphorus was estimated by King's (1932) method after heating a sample of the acid extract of the ash with a few drops of conc. H_2SO_4 to convert any pyrophosphate into orthophosphate. This method gave results in close agreement with those obtained by a wet-digestion method.

Chloride was estimated on duplicate samples of about 1 g. of tissue by heating in a boiling-water bath with 5 ml. of 0·02N-$AgNO_3$ and 5 ml. of conc. HNO_3 and titrating the excess of $AgNO_3$ with 0·02N-KSCN in the presence of $Fe_2(SO_4)_3$. Samples for nitrogen determination were weighed and transferred to Universal containers (Baird and Tatlock Ltd.), where they were covered with conc. H_2SO_4 and left to disintegrate. This took about a week at room temperature, but the process was hastened by heating in an oven at 100° overnight. The mixture was made up to volume with water in a 50 or 100 ml. graduated flask and a portion (5 or 10 ml.) of the fine suspension taken for micro-Kjeldahl determination. The sample was digested with 2 ml. of conc. H_2SO_4, with copper selenite as catalyst. Fat was estimated on a sample of dried material by extraction with light petroleum.

Portions (about 2·5 g.) of muscle, liver and heart of the newborn piglet to which inulin had been given, and of its control, were covered with water and heated in a boiling-water bath for 10 min. to destroy enzymic activity, and ground with sand. The mixture was transferred to a 50 ml. graduated cylinder and allowed to cool. Zinc sulphate solution (5 ml.; 10%, w/v) and 5 ml. of 0·5N-NaOH were added to precipitate the protein. The contents of the cylinder were made up to a known volume and filtered. A portion of the protein-free filtrate (2 ml.) was extracted three times with redistilled butan-2-ol, the volumes of aqueous and non-aqueous phases being adjusted to give a ratio of 95:1 between non-aqueous and aqueous volumes after mixing. This process was carried out in order to remove the bulk of the extraneous substances giving a colour in the method used for the determination of fructose. The aqueous phase was then warmed to drive off excess of solvent and made to a known volume, and a portion taken for inulin determination. The estimation of fructose was made by the method of Cole as described by Bacon & Bell (1948), but special precautions were taken to exclude light during colour development as exposure to bright light was found to reduce the intensity of the colour produced.

Inulin was determined in the serum after deproteinizing with $ZnSO_4$ and NaOH, and partitioning with butanol by the same procedure as that described for other tissues. Inulin in urine was determined directly on diluted urine.

Chloride in serum was determined by Sendroy's (1937) technique, and sodium with the Beckman flame photometer. A portion (0·2 ml.) of serum was added to 2 ml. of 10% trichloroacetic acid to precipitate the proteins; the mixture was centrifuged and 1 ml. of the supernatant fluid diluted to 10 ml. for the sodium determination.

RESULTS

Table 1 shows the effect of bleeding on the composition of the skeletal muscle of the rat, fowl, pig and man. The concentration of chloride was much lower in the muscles of the cockerel, pigs and men which had lost a great deal of blood before they died than in the muscles of those which had not. In pigs, for example, the difference was 9 m-equiv./kg. and this could not have been due simply to a loss of blood serum from the muscle, for it would have meant that unbled animals' muscle contained over 90 ml. of serum or 150 ml. of blood/kg., which it clearly did not. It is evident that extracellular fluids had been withdrawn from the muscle into the circulation while the bleeding was going on, and this is confirmed by the figures for sodium in the pig and man. There was no such difference in the rat. This was no doubt partly because a much smaller proportion of the total blood volume was taken from the rats than from the cockerels or pigs, but in fact we found it impossible to remove more than about one-third of the blood volume of a rat by bleeding from the abdominal aorta or by decapitation. White & Rolf (1955, 1956) do not seem to have had this difficulty, but in view of the rapidity with which rat's blood clots it seems as though the term 'exsanguination' as applied by various workers to rats may well not mean the same as it does in other species. It would be interesting to repeat this experiment with heparinized animals.

As might have been expected, the comparison of the composition of rat muscle obtained by biopsy with that of muscle obtained at autopsy without bleeding did not show any significant differences, and the results have therefore not been presented.

The concentrations of the cellular ions, potassium and phosphate, tended to be slightly higher in the muscle obtained after bleeding, but these may have been chance variations where the 'unbled' and 'bled' samples came from different animals or men. There were no consistent differences in the water or nitrogen. This is surprising, for a loss of extracellular fluid from the muscle must have resulted in a lower percentage of water in the tissue, and this anomalous result must have been due to individual variations and the small number of samples analysed.

The thigh muscle of the control newborn piglet contained 83·8% of water and 36 m-equiv. of chloride/kg. before 20 ml. of blood was removed

Table 1. *Effect of bleeding on the composition of skeletal muscle*

Results are expressed on a fat-free basis.

	Rat		Fowl		Pig		Man	
	Biopsy before bleeding	Autopsy from other side after removal of 5–8 ml. of blood	Killed without bleeding	Decapitated and exsanguinated	Killed without bleeding	Exsanguinated at slaughterhouse	Little or no bleeding before death	Severe bleeding
No. of observations	5	5	1	1	2	4	2	2
Water (g./100 g.)	77·8 (77·5–78·2)	77·5 (77·2–77·8)	74·1	74·1	77·8	76·0 (75·6–76·8)	77·5	79·5
N (g./100 g.)	3·20 (3·10–3·30)	3·26 (3·24–3·30)	3·89	3·76	3·30	3·61 (3·51–3·71)	3·36	3·06
Cl (m-equiv./kg.)	19·6 (17·8–23·9)	19·4 (16·8–20·6)	20·6	14·7	22·4	13·4 (12·8–14·6)	22·6	17·9
Na (m-equiv./kg.)	23·7 (23·4–24·0)	23·6 (22·5–24·2)	—	—	26·6	19·4 (18·7–21·1)	30·8	24·0
K (m-equiv./kg.)	121 (117–124)	124 (121–126)	108	108	98	103 (97–109)	105	111
P (mg./100 g.)	245 (239–252)	259 (254–265)	242	245	222	227 (215–234)	201	205

Table 2. *Concentration of inulin, chloride and sodium in the serum and tissues of the experimental piglet, and the inulin, chloride and sodium 'spaces'*

	Serum	Muscle	Heart	Liver
Water (g./100 g.)	—	82·8	80·9	79·0
Inulin (mg./100 g.)	17·8	5·06	5·46	11·8
Cl (m-equiv./kg.)	103	34·5	41·0	36·5
Na (m-equiv./kg.)	140	46·5	—	45·5
Inulin 'space' (ml./100 g.)	—	28·0	30·2	64·9
Chloride 'space' (ml./100 g.)	—	31·0	37·1	32·8
Sodium 'space' (ml./100 g.)	—	33·9	—	33·5

from the heart, and 82·8 % of water and 33·5 m-equiv. of chloride/kg. afterwards. It would appear that the removal of 20 ml. of blood, or about one-sixth of the blood volume, from a newborn pig makes a measurable difference to the concentration of chloride in its muscle. Hence there may be a real difference between the response of rat muscle to haemorrhage and the response of the muscles of other animals, but work on more animals is needed to establish this point.

Table 2 shows the concentration of 'true inulin' and of chloride and sodium in the serum, skeletal muscle, heart and liver of the experimental piglet. The urine excreted by the experimental piglet contained 564 mg. of inulin, which was two-thirds of the dose injected. The concentration of inulin in the liver was over twice that in the skeletal muscle or heart, whereas the concentration of chloride was of the same order in all three tissues. Table 2 also shows the inulin 'space', chloride 'space' and the sodium 'space' in the same three tissues, calculated in the conventional way. The chloride space was a little higher than the inulin space in skeletal muscle but some such difference was to be expected from work on whole animals (Gaudino, Schwartz & Levitt, 1948; Gamble & Robertson, 1952). The same is true of the results for the heart, although the difference was a little greater. The sodium space corresponded closely to the chloride space. The inulin space in the liver, however, was twice that of the chloride space. This was due to the high concentration of inulin found there, and not to a low concentration of chloride, for the latter was similar to the value obtained on many other newborn piglets which were used for other investigations.

DISCUSSION

Effect of haemorrhage

The results of the present investigation indicate that bleeding materially reduces the concentration of chlorides in skeletal muscle, at any rate in some species. It seems important to recognize this, and it is in fact evident from the literature, for some authors have bled their animals to death, others have removed enough blood for analysis, whereas others have not removed any blood at all, and the figures for chlorides obtained by them all are in general agreement with the concept now put forward. The differences due to bleeding in man, the pig and the fowl, and probably also in the dog and cat, may be quantitatively much greater than differences due to fat, connective tissue or contaminating blood. If the chloride space is used only to calculate the composition of the intracellular compartment it probably will not make much difference to the conclusions reached whether or not the animals have been bled. It must, however, make a great difference to any calculation of the relationship of cell mass to extracellular water.

Inulin and chloride spaces

The present results confirm those of others that the chloride space in skeletal muscle is somewhat greater than the inulin space (Wilde, 1945; Cotlove, 1952; Mokotoff, Ross & Leiter, 1952; Nichols *et al.* 1953), and it is clear that the newborn pig does not have large quantities of chloride in its muscle cells. White & Rolf (1956) reported that the liver of the adult rat also 'holds up' inulin and they found that the same applied to skin and spleen. They suggested that macrophages in these three tissues actively ingest and concentrate inulin. This hold-up of inulin, probably within cells, clearly makes it useless as a guide to the extracellular space in these organs.

SUMMARY

1. The concentrations of chloride and of sodium in the skeletal muscle of man, the pig and the fowl are reduced by about one-third if severe bleeding takes place before death. The rat does not react in this way; this may be due partly to the fact that rats do not bleed as freely as other, larger animals, but there may be a further species difference.

2. The gross composition of the cells is probably little altered by bleeding.

3. The inulin 'space' in the skeletal and cardiac muscle of a newborn pig was a little less than the chloride 'space' but it was more than twice as large in the liver; inulin cannot be taken to measure the extracellular space in that organ.

The results presented in this paper have been obtained in the course of several different investigations in which a number of people have been involved. We wish to thank them all, and particularly Professor R. A. McCance for his help with the presentation of the results. The method for the estimation of inulin in tissues was developed by Dr W. I. M. Holman, and the figures for the fowl were given us by Mr J. W. T. Dickerson.

REFERENCES

Bacon, J. S. D. & Bell, D. J. (1948). *Biochem. J.* **42**, 397.
Barnes, B. A., Gordon, E. B. & Cope, O. (1957). *J. clin. Invest.* **36**, 1239.
Bernstein, R. E. (1952). *Biochim. biophys. Acta*, **9**, 576.
Cotlove, E. (1952). *Fed. Proc.* **11**, 28.
Crismon, J. M. (1948). *Fed. Proc.* **7**, 24.
Darrow, D. C., Harrison, H. E. & Taffel, M. (1939). *J. biol. Chem.* **130**, 487.
Gamble, J. L. jun. & Robertson, J. S. (1952). *Amer. J. Physiol.* **171**, 659.
Gaudino, M., Schwartz, J. L. & Levitt, M. F. (1948). *Proc. Soc. exp. Biol., N.Y.*, **68**, 507.
Hines, H. M. & Knowlton, G. C. (1939). *Proc. Soc. exp. Biol., N.Y.*, **42**, 133.
Kerpel-Fronius, E. (1937). *Z. Kinderheilk.* **58**, 726.
King, E. J. (1932). *Biochem. J.* **26**, 292.
Lowry, O. H., Hastings, A. B., Hull, T. Z. & Brown, A. N. (1942). *J. biol. Chem.* **143**, 271.
McCance, R. A. & Shipp, H. L. (1933). *Spec. Rep. Ser. med. Res. Coun., Lond.*, no. 187.
McCance, R. A., Widdowson, E. M. & Shackleton, L. R. B. (1936). *Spec. Rep. Ser. med. Res. Coun., Lond.*, no. 213.
Manery, J. F. & Hastings, A. B. (1939). *J. biol. Chem.* **127**, 657.
Mokotoff, R., Ross, G. & Leiter, L. (1952). *J. clin. Invest.* **31**, 291.
Mudge, G. H. & Vislocky, K. (1949). *J. clin. Invest.* **28**, 482.
Nichols, G. jun., Nichols, N., Weil, W. B. & Wallace, W. M. (1953). *J. clin. Invest.* **32**, 1299.
Sendroy, J. (1937). *J. biol. Chem.* **120**, 405.
Talso, P. J., Spafford, N. & Blaw, M. (1953). *J. Lab. clin. Med.* **41**, 405.
White, H. L. & Rolf, D. (1955). *Amer. J. Physiol.* **180**, 287.
White, H. L. & Rolf, D. (1956). *Amer. J. Physiol.* **185**, 152.
Wilde, W. S. (1945). *Amer. J. Physiol.* **143**, 666.
Wilson, A. O. (1955). *Brit. J. Surg.* **43**, 71.

The Effect of Chlorpromazine on the Respiratory Chain

CYTOCHROME OXIDASE

By M. J. R. DAWKINS,* J. D. JUDAH and K. R. REES
Department of Morbid Anatomy, University College Hospital Medical School and Biochemistry Department, University College London

(*Received* 17 *September* 1958)

The phenothiazine 'tranquillizer' chlorpromazine [10(3-dimethylaminopropyl)-2-chlorphenothiazine hydrochloride] has a powerful depressant action on the central nervous system. In view of the inhibitory effects of the barbiturates on electron transport (Ernster, 1956), we have investigated the effects of chlorpromazine on the respiratory chain. The work of Berger, Strecker & Waelsch (1956) has shown that chlorpromazine inhibited the phosphorylation coupled to the oxidation of cytochrome *c*. This paper is concerned with a detailed analysis of this inhibition.

METHODS

Wistar albino rats fed on an unrestricted M.R.C. diet no. 41 (Bruce, 1950) were used throughout the investigation. Mitochondria were prepared from liver by the method of Schneider (1948) and from brain as described by Christie, Judah & Rees (1953). Mitochondria were depleted of pyridine nucleotide by pre-incubation in 0·25 M-sucrose–0·025 M-sodium orthophosphate–HCl buffer, pH 7·2, for 10 min. at 30° before use, as described by Hunter & Ford (1955). The phosphorylation coupled to the oxidation of ferrocytochrome *c* was studied with mitochondria suspended in 0·25 M-sucrose as described by Judah (1951) or suspended in 0·075 M-sucrose as described by Lehninger, ul Hassan & Sudduth (1954). Phosphorylation coupled to the reduction of cytochrome *c* by L-glutamate and β-hydroxybutyrate was studied by the method of Borgstrom, Sudduth & Lehninger (1955). The reduction of cytochrome *c* was followed spectrophotometrically with the Unicam SP. 500 spectrophotometer by measuring the increase in absorption at 550 mμ. Phosphorylation was measured by the method described by Nielsen & Lehninger (1955).

Oxidative phosphorylation with oxygen as electron acceptor was studied as described by Judah (1951). The final concentrations of the components were: adenosine triphosphate (ATP), 1·7 mM; $MgSO_4$, 6·7 mM; KCl, 25 mM; sodium orthophosphate–HCl buffer, pH 7·2, 10 mM; cytochrome *c*, 10 μM; glucose, 30 mM; purified yeast hexokinase added in 0·05 ml.; substrate 10 mM; NaF, 13 mM. Mitochondria were added in 0·25 M-sucrose as indicated in the text. Final volume was 3 ml. Incubation temperatures are indicated for each experiment. The gas phase was air.

Ethylenediaminetetra-acetate (EDTA) was added as indicated in all experiments with cytochrome oxidase in

* Stothert Research Fellow of the Royal Society.

order to decrease the metal-catalysed auto-oxidation of ascorbate. All oxygen uptakes quoted are corrected for this. EDTA was also included in experiments in which small quantities of enzyme were used, e.g. spectrophotometric experiments.

MATERIALS

All nucleotides and cytochrome *c* were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A.

Radioactive phosphate was obtained from The Radiochemical Centre, Amersham, Bucks, and purified as described by Kennedy (1953). Chlorpromazine was a gift from May and Baker Ltd., Dagenham, Essex. Yeast hexokinase was prepared by the method of Berger, Slein, Colowick & Cori (1945). It was purified to the stage of fraction 3 of their procedure. The conditions for autolysis of baker's yeast described by Berger *et al.* (1945) were found to be unsatisfactory and those described by Bailey & Webb (1948) were used instead.

RESULTS

Action on phosphorylating system. When chlorpromazine at concentrations of 0·2 mM is added to suspensions of respiring liver mitochondria, with β-hydroxybutyrate and succinate as substrates, reduction of the efficiency of the coupled phosphorylation invariably occurs (Table 1). In about 30% of the experiments with β-hydroxybutyrate as substrate, the oxygen uptake was inhibited 50% or more. In these experiments, the P/O ratios were the same as in those in which no respiratory inhibition was produced by chlorpromazine. The reduction of phosphorylation is far greater with succinate as substrate. This result suggests that chlorpromazine may be acting at a single phosphorylating site. The only one common to both these substrates is that involved in the oxidation of ferrocytochrome *c*. Inhibition of this step would have a disproportionately greater effect on the P/O ratio for succinate (P/O 2) than on the P/O ratio for diphosphopyridine nucleotide (DPN)-linked substrates (P/O 3).

We found that 0·1 mM-chlorpromazine inhibited phosphorylation coupled to the oxidation of ferrocytochrome *c* by upwards of 70%. Table 2 shows that this result could be obtained when the mitochondria were suspended in 0·25 M-sucrose or 0·075 M-sucrose and at concentrations of cytochrome *c* between 0·01 and 0·06 mM. The small uptakes of inorganic phosphate occasionally observed in the presence of 0·2 mM-chlorpromazine are not considered significant. It is clear that the phosphorylation is essentially nil.

We also tested the effect of chlorpromazine on mitochondria reducing cytochrome *c* in the presence of β-hydroxybutyrate and L-glutamate. The method of Borgstrom *et al.* (1955), which we used, requires a pretreatment of the mitochondria with hypotonic sucrose solution in order to make the added cytochrome *c* available to the electron-transport chain

Table 1. *Effect of chlorpromazine on phosphorylation coupled to oxidation of β-hydroxybutyrate and succinate*

Contents of the flasks were as described in Methods. Mitochondria equivalent to 1 mg. of N were added to each flask in 0·5 ml. of 0·25 M-sucrose. Temperature of the bath was 38° and the final volume of the flask contents was 3 ml.

Substrate	Concn. of chlorpromazine (mM)	O_2 uptake (μatoms)	P uptake (μmoles)	P/O ratio	Inhibition of P/O ratio (%)
Succinate	Nil	12·5	21	1·7	Nil
Succinate	0·2	15·0	12	0·8	53
β-Hydroxybutyrate	Nil	5·3	15·5	2·9	Nil
β-Hydroxybutyrate	0·2	5·3	12	2·3	21

Table 2. *Action of chlorpromazine on phosphorylation coupled to oxidation of ferrocytochrome* c

Flasks contained: cytochrome *c*, as indicated; ascorbate, 10 mM; neutralized to pH 7·0; EDTA, final concn. 0·3 mM; other components were as described in Methods except that no substrate other than ascorbate and cytochrome *c* was added. Final volume, 3 ml. Mitochondria equivalent to 2 mg. of N were added. Temperature of the bath was 20°. The results are corrected for blank values in the absence of cytochrome *c*. They are representative of 10 such experiments.

Mitochondria in	Concn. of cytochrome *c* (μM)	Concn. of chlorpromazine (mM)	O_2 uptake (μatoms)	P uptake (μmoles)	P/O	Inhibition of P/O ratio (%)
0·25 M-Sucrose	60	Nil	11·2	4	0·36	Nil
	60	0·1	10·8	1	0·09	75
	20	Nil	7·8	3·5	0·45	Nil
	20	0·1	8·2	Nil	—	100
0·075 M-Sucrose	10	Nil	10·9	6·5	0·6	Nil
	10	0·1	9·0	1·5	0·17	72

Table 3. *Effect of chlorpromazine on reduction of cytochrome* c *and coupled phosphorylation*

Components were as described in Methods. EDTA (0·3 mM) was added; mitochondria in 0·075 M-sucrose, equivalent to 0·04 mg. of N, were added to the cuvettes to start the reaction. Final volume, 3 ml. Temperature, 20°. Figures in the table are corrected for the appropriate blanks without added substrate. The expression P/2E is defined as the ratio of μm-moles of inorganic orthophosphate esterified per pair of μm-moles of cytochrome *c* reduced, since this is a one-electron transfer.

Substrate	Concn. of chlorpromazine (mM)	Time of incubation (min.)	Cytochrome *c* reduced (μm-moles)	Inorganic P esterified (μm-moles)	P/2E	Inhibition of reduction (%)
L-Glutamate	Nil	11	27·0	21·5	1·6	—
	0·1	16	20·0	15·8	1·58	50
β-Hydroxybutyrate	Nil	10	12·0	7·8	1·3	—
	0·1	15	8·2	6·5	1·58	54

Table 4. *Effect of chlorpromazine on phosphate-treated mitochondria oxidizing succinate in the presence and the absence of cytochrome* c

Components were as described in Methods. Mitochondria equivalent to 0·5 mg. of N were added in 0·5 ml. of 0·25 M-sucrose–0·02 M-sodium orthophosphate–HCl buffer (pH 7·15). Final volume, 3 ml. Temperature of the bath, 38°. These results are typical of four such experiments.

Cytochrome *c* (20 μM)	Concn. of chlorpromazine (mM)	O_2 uptake (μatoms)	P uptake (μmoles)	P/O	Inhibition of P/O ratio (%)
–	Nil	6·0	9·0	1·5	Nil
+	Nil	15·6	24	1·5	Nil
–	0·1	6·3	7	1·1	27
+	0·1	16·3	14·0	0·8	43

of the phosphorylating system. Chlorpromazine was added at a concentration of 0·1 mM, since preliminary experiment showed that under these conditions gross inhibitions of electron transport were encountered at higher concentrations. Table 3 shows that 0·1 mM-chlorpromazine gave some 50 % inhibition of the rate of cytochrome *c* reduction. The inhibited systems were allowed to run longer to achieve reasonably comparable figures, since under the conditions of these experiments the reduction of cytochrome *c* and the esterification of inorganic phosphate were linear with time. There was no reduction in the efficiency of the phosphorylating process, which is expressed in terms of moles of inorganic orthophosphate esterified per pair of electrons passing to cytochrome *c*. It may be concluded therefore that the inhibitor is relatively specific for the phosphorylation coupled to the terminal step of electron transport.

Further information on the action of chlorpromazine was obtained from a study of phosphate-treated mitochondria. Hunter & Ford (1955) described this technique for depleting mitochondria of DPN. Such mitochondria show pronounced requirements for DPN and are able to carry out oxidative phosphorylation only upon addition of substrate and DPN. We investigated these phosphate-treated particles and found that they also showed considerable stimulation of oxygen uptake with succinate as substrate, on the addition of cytochrome *c*. The extra oxygen uptake is coupled to phosphorylation, the P/O ratios being similar

Table 5. *Effect of adenosine triphosphate, magnesium ions, potassium ions and cytochrome* c *on ferrocytochrome* c *oxidation*

Flasks contained: sodium phosphate buffer (10 mM), pH 7·2; ascorbate, 10 mM, pH 7·0; EDTA, 0·3 mM; mitochondria equivalent to 1 mg. of N were added in 0·25 M-sucrose. Temperature of the bath, 38°. Final volume of the flask contents was 3 ml.

Additions	O_2 uptake (μl./10 min.)
Cytochrome *c* (20 μM)	68
Cytochrome *c* (40 μM)	136
Cytochrome *c* (40 μM) + ATP (1·7 mM)	78
Cytochrome *c* (40 μM) + ATP (1·7 mM) + KCl (25 mM) + Mg^{2+} (6·7 mM)	17
Cytochrome *c* (40 μM) + KCl (25 mM) + Mg^{2+} (6·7 mM)	60

both in the presence and the absence of added cytochrome *c*. Table 4 shows that chlorpromazine inhibits both systems, but the inhibition in the presence of added cytochrome *c* is 43 % and only 27 % in its absence, suggesting that the inhibitor acts less effectively upon the bound as opposed to the added protein.

Action of chlorpromazine on ferrocytochrome c *oxidation.* In the systems used for studying the coupled phosphorylation, the oxidation of ferrocytochrome *c* does not proceed at maximal rates. This is due to several factors, amongst which are the low levels of cytochrome *c* added, and probably the accessibility of the protein to the oxidase system. Table 5 shows the importance of these

points. Thus the level of cytochrome *c* usually employed in the phosphorylating system (0·02 mM) permits very much less than the maximal rate, and addition of ATP, Mg^{2+} and K^+ ions reduces the rate still further. If the ATP is hydrolysed in N-HCl for 10 min. and then neutralized its inhibitory action is removed; this suggests that its action is due to maintenance of the normal mitochondrial permeability, which thus prevents access of added cytochrome *c*.

We therefore tested the effect of 0·2 mM-chlorpromazine in mitochondrial systems where such barriers do not exist. This was achieved by the use of water suspensions of mitochondria where no coupled phosphorylation took place. Table 6 shows that an 89% inhibition of the cytochrome oxidase system occurred under these conditions. The inhibition was dependent upon the concentration of added cytochrome *c*, falling to 18% when the cytochrome *c* was increased to 0·2 mM.

This result may be due to binding of the inhibitor by the protein, and in order to exclude this possibility the three following tests were performed.

Nature of the inhibition of cytochrome oxidase. Analysis by the method of Lineweaver & Burk (1934) was carried out with three concentrations of chlorpromazine and four concentrations of cytochrome *c*. Fig. 1 shows the results of one such experiment, which is typical of several. It will be observed that curves are obtained which meet at a common intercept when extrapolated to infinite substrate concentration. This indicates competitive inhibition, and makes it unlikely that simple binding is the cause of the reversal of chlorpromazine inhibition by added cytochrome *c*. Nevertheless, further experiments were performed to confirm this point. Use was made of two other properties of chlorpromazine, one being that the substance at a concentration of 0·1 mM will inhibit powerfully the oxidation of β-hydroxybutyrate, provided that the mitochondria have been subjected to hypotonic treatment in order to permit access of the inhibitor. The other function tested was the inhibition of oxidative phosphorylation with succinate as substrate. In this latter test, phosphate-treated mitochondria were again used, this time to ensure a one-step oxidation of succinate, since the successive oxidations cannot occur in mitochondria depleted of DPN. Neither of these actions of chlorpromazine was in the least affected by the presence of cytochrome *c* (up to 0·2 mM), and it may therefore be concluded that simple binding of the inhibitor by the protein does not occur.

Action of chlorpromazine on succinate oxidation. Elsewhere we have noted that both succinate and β-hydroxybutyrate oxidations may be powerfully inhibited by chlorpromazine (Dawkins, Judah & Rees, 1959, unpublished work). However, we have observed that water suspensions of mitochondria oxidizing succinate may show considerable acceleration of respiration in the presence of 0·1 mM-chlorpromazine. Table 7 shows this effect on succinate

Table 6. *Inhibition of cytochrome oxidase by chlorpromazine*

Flasks contained: sodium phosphate buffer (16 mM), pH 7·2; ascorbate, 10 mM; EDTA, 0·3 mM; mitochondria in water equivalent to 0·2 mg. of N were added to each flask. Temperature of the bath, 38°. Final volume of the flask contents was 3 ml.

Concn. of cytochrome *c* (μM)	O_2 uptake (μl./5 min.) Control	Chlorpromazine (0·2 mM)	Inhibition by chlorpromazine (%)
20	45	5	89
67	72	23	68
130	79	52	34
200	79	65	18

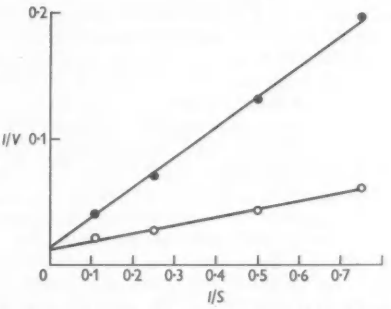


Fig. 1. Demonstration of competitive inhibition between chlorpromazine and cytochrome *c*. The cytochrome *c* concentrations (*S*) were 200, 89, 40 and 30 μM. The conditions of the reaction were as described in Table 6. Concentrations of chlorpromazine were 0·15 mM (○) and 0·2 mM (●). *V* is the oxygen uptake in the first 10 min. Temperature was 38°.

Table 7. *Acceleration of succinate oxidation by chlorpromazine*

Flasks contained: sodium phosphate buffer (17 mM), pH 7·2; cytochrome *c*, as indicated. Mitochondria in water equivalent to 0·2 mg. of N were added. Final volume of the flask contents was 3 ml. Temperature of the bath was 38° and the time of incubation 20 min.

	O_2 uptake (μl.)		
Concn. of cytochrome *c* (μM) ...	20	100	200
Additions			
Succinate (33 mM)	82	82	88
Succinate (33 mM) + chlorpromazine 0·1 (mM)	117	121	122

Table 8. *Action of chlorpromazine on brain mitochondria*

The succinate system was as described in Methods. Mitochondria were suspended in 0·25 M-sucrose and the incubation temperature was 38°. The ferrocytochrome *c* system was as described in Table 2. Mitochondria were suspended in 0·075 M-sucrose and the incubation temperature was 20°. Final volume of flask contents was 3 ml.

Substrate	Concn. of chlorpromazine (mM)	O_2 uptake (μatoms)	P uptake (μmoles)	P/O	Inhibition of P/O (%)
Succinate	Nil	8·6	10·0	1·2	Nil
Succinate	0·2	6·1	3·5	0·57	53
Ferrocytochrome *c*	Nil	5·0	4	0·80	Nil
Ferrocytochrome *c*	0·1	6·2	3·0	0·48	40
Ferrocytochrome *c*	0·2	3·3	0·0	—	100

oxidation at three concentrations of cytochrome *c*. It seems that both control and accelerated rates are not influenced by variation in cytochrome concentration. Quite probably this result reflects a structural effect on the particles, since it is difficult to see how such an acceleration could be brought about by other means in this system.

Action of chlorpromazine on brain systems. Berger *et al.* (1956) were unable to detect any uncoupling effect of chlorpromazine on brain preparations. Table 8 shows typical effects of chlorpromazine on brain mitochondria. Thus there is 50 % inhibition of the P/O ratio in the presence of 0·2 mM-chlorpromazine when succinate is the substrate. In addition, there is a very distinct inhibition of cytochrome oxidase, though this is not so marked as with liver preparations. Finally, there is a clear-cut inhibition of the phosphorylation coupled to ferrocytochrome *c* oxidation.

It thus appears that there is no special difference between brain and liver preparations in their sensitivity to chlorpromazine, but it is true that brain-mitochondrial preparations are far from homogeneous, and it may be that the results of Berger *et al.* (1956) were due to the presence of extraneous matter which might well bind the inhibitor.

DISCUSSION

The fact that Berger *et al.* (1956) observed no inhibition of respiration by chlorpromazine may be ascribed to several factors which are pertinent to this discussion. In the first place, the systems they used for determining the phosphorylation coupled to the oxidation of ferrocytochrome *c* do not absorb oxygen at maximum rate. We have found in investigating this point that rates of the order of 20 % of maximal are in fact observed. This in turn is due to several factors. The concentration of added cytochrome *c* used by Berger *et al.* was kept below the optimum; and it also happens that the presence of ATP, K^+ ions and Mg^{2+} ions exert a powerful inhibitory effect, presumably by influencing mitochondrial permeability to cytochrome *c*. Our results show that powerful inhibitions of the oxidase system may be obtained under the correct conditions. The nature of the inhibition remains in doubt. However, the apparent competitive nature of the inhibition does suggest that interaction between ferrocytochrome *c* and the next component, probably cytochrome *a*, is being disturbed, in which case one would expect reduction of cytochrome *c* and oxidation of cytochromes *a* and a_3 to occur during chlorpromazine inhibition of the system.

The fact that inhibition of the phosphorylation is not relieved by added cytochrome *c* is of interest, since it suggests that the inhibitor is acting at two sites both related to cytochrome *c*. One possibility that should be borne in mind is that some non-phosphorylating pathway for the oxidation of ferrocytochrome *c* is being brought into play by chlorpromazine.

Our experiments with phosphate-treated mitochondria oxidizing succinate in the presence and the absence of added cytochrome *c* showed that the inhibition of phosphorylation by chlorpromazine in the former case was some 50 %, whereas in the latter it was only some 20 %. This system made it possible for us to perform an experiment in which added cytochrome *c* induced a large excess of oxygen uptake, which was coupled to a phosphorylation, and in which the behaviour of the internally bound cytochrome *c* could also be studied, and it would appear that chlorpromazine is a more effective uncoupler of phosphorylation accompanying the oxidation of added ferrocytochrome *c* than of the phosphorylation associated with the oxidation of the endogenous protein.

The apparent specificity of chlorpromazine in inhibiting the phosphorylation coupled to ferrocytochrome *c* oxidation needs some discussion. We have shown that a concentration of chlorpromazine which brings about 50 % inhibition of electron transport between substrate and cytochrome *c* (Table 3) reduces the phosphorylation coupled with this process by the same amount, leaving the P/O ratio unaltered. Such a result does not necessarily mean that one phosphorylation is basically different from the others, it may merely

be more sensitive. Lehninger *et al.* (1954) noted that the phosphorylation coupled to the oxidation of ferrocytochrome *c* was rather more sensitive to common uncoupling agents than other steps.

We think that the specificity of chlorpromazine is a true one, since studies of other phenothiazines reveal a similar action to that of chlorpromazine on the phosphorylation coupled to the oxidation of ferrocytochrome *c*. In addition, at least one compound also uncouples the phosphorylation coupled to reduction of cytochrome *c* by succinate, but has no effect on the efficiency of the phosphorylation coupled to the reduction of cytochrome *c* by β-hydroxybutyrate (Dawkins, Judah & Rees, 1959, unpublished work).

A question which clearly must be raised is whether these actions of chlorpromazine can account for its biological effect? The inhibition of oxidative phosphorylation is, at its maximum, rather small—it cannot be greater than some 33% overall—and it is hard to see how this could bring about any pronounced effect on the organism. Conceivably, the inhibition of electron transport might be of importance. In this connexion, our results with brain mitochondria, though they are similar to those with liver, do not indicate any special sensitivity to the drug. If anything, there is rather less effect on brain mitochondria.

The concentration of cytochrome *c* in whole cells is probably lower than in our artificial systems. An increase in the concentration of cytochrome *c* reduces the inhibition of cytochrome oxidase by chlorpromazine. Thus the high (by pharmacological standards) concentrations of chlorpromazine which we found necessary to inhibit cytochrome oxidase may only reflect the high concentrations of cytochrome *c* in our test system. Possibly there is some sensitive site *in vivo* which specifically responds to low concentrations of chlorpromazine. Investigations are now proceeding into other agents of the phenothiazine group and into related compounds to see whether they affect tissues in the same way as chlorpromazine.

SUMMARY

1. Chlorpromazine at a concentration of 0·1 mM has been shown to uncouple the phosphorylation coupled to the oxidation of reduced cytochrome *c*. It does not affect the phosphorylations coupled to the previous steps in the electron-transport chain.

2. Chlorpromazine inhibits cytochrome oxidase; this inhibition is dependent on both chlorpromazine and cytochrome *c* concentrations.

3. Analysis of the nature of the inhibition suggests that it is competitive.

4. These effects were found in mitochondria isolated both from brain and liver.

We should like to thank Sir Roy Cameron, F.R.S., for his constant interest and Dr J. F. Smith for helpful discussions. This work was supported by grants to one of us (J.D.J.) by the Medical Research Council and by the Graham Research Fund of the University of London.

REFERENCES

Bailey, K. & Webb, E. C. (1948). *Biochem. J.* **42**, 60.
Berger, L., Slein, M. W., Colowick, S. P. & Cori, C. F. (1945). *J. gen. Physiol.* **29**, 379.
Berger, M., Strecker, H. J. & Waelsch, H. (1956). *Nature, Lond.*, **177**, 1234.
Borgstrom, B., Sudduth, H. C. & Lehninger, A. L. (1955). *J. biol. Chem.* **215**, 571.
Bruce, H. M. (1950). *J. Hyg., Camb.*, **48**, 171.
Christie, G. S., Judah, J. D. & Rees, K. R. (1953). *Proc. Roy. Soc.* B, **141**, 523.
Ernster, L. (1956). *Exp. cell. Res.* **10**, 721.
Hunter, F. E. & Ford, L. (1955). *J. biol. Chem.* **216**, 357.
Judah, J. D. (1951). *Biochem. J.* **49**, 271.
Kennedy, E. P. (1953). *J. biol. Chem.* **201**, 399.
Lehninger, A. L., ul Hassan, M. & Sudduth, H. C. (1954). *J. biol. Chem.* **210**, 910.
Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
Nielsen, S. O. & Lehninger, A. L. (1955). *J. biol. Chem.* **215**, 555.
Schneider, W. C. (1948). *J. biol. Chem.* **176**, 259.

Acetylcholinesterase in Goldfish Muscles

STUDIES ON SOME SUBSTRATES AND INHIBITORS

By S. J. LUNDIN

Research Institute of National Defence, Department 1, *Sundbyberg* 4, *Sweden*

(*Received* 28 *August* 1958)

In an earlier paper (Lundin, 1958) it was pointed out that the cholinesterase activity in muscles of goldfish (*Carassius auratus*) and guppy (*Lebistes reticulatus*) is due to an acetylcholinesterase. Working with goldfish muscles the author has obtained a high proportion of acetylcholinesterase activity in the supernatant after centrifuging the homogenized muscles. This preparation of acetylcholinesterase has been found to be very stable and is convenient for various purposes, e.g. routine tests of organic phosphorus compounds or kinetic enzyme studies. Thus it seemed to be of interest to give a detailed account of the results on which is based the statement that goldfish muscles contain acetylcholinesterase. It is well known (e.g. Augustinsson, 1949) that substrate and inhibitor relationships of acetylcholinesterases from different sources differ. A study of these relationships of the goldfish-enzyme preparation to some important substrates and inhibitors as compared with those of some other well-known sources has therefore been undertaken.

METHODS AND MATERIALS

Enzyme activities were measured by Tammelin's (1953) modification of Michel's (1949) electrometric method. The activities are expressed as μm-moles of acid liberated ml.$^{-1}$ min.$^{-1}$, after correction for the spontaneous hydrolysis of the substrates as measured in individual blanks in the presence of suitable concentrations of the denatured (heated to 100°) enzymes. The highest and lowest values obtained for the spontaneous hydrolysis are given in Table 1.

Buffers used

A. A buffer of the following composition and of ionic strength 0·25 was found to be the best to extract the acetylcholinesterase from the muscles of goldfish: 0·01 M-sodium barbital, 0·002 M-KH_2PO_4, 0·228 M-KCl. 0·1 N-HCl was added to obtain pH 8·2. The pH was measured with a pH meter, PHM 3 from Radiometer, Copenhagen, against a standard buffer at pH 7 (P-H Tamm, Altuna, Sweden).

B. Activity measurements of the fish enzyme preparations were carried out in a buffer of the same composition as *A*, except that the concentration of KCl was 0·3 M.

C. Erythrocyte and plasma cholinesterase activities were measured in a buffer of twice the concentration of *B*.

Sources of cholinesterases

From goldfish. Freeze-dried body muscles from goldfish were homogenized in buffer *A*. The homogenate was centrifuged for 15 min. at 1500 *g* and the supernatant was dialysed for 24 hr. against distilled water and freeze-dried once more. This preparation gave very uniform and stable activities when redissolved in buffer *A* (10 mg./ml.). The solution was diluted 30 times with buffer *B* before use.

From electric ray. A freeze-dried preparation of the electric organ of electric ray (*Torpedo ocellata*) was homogenized in *B* (0·3 g./100 ml.) and was centrifuged for 1 min. at about 1000 *g*. The almost clear supernatant was used.

From erythrocytes. Fresh, heparinized human blood was centrifuged for 15 min. at 1500 *g* and the plasma discarded. The remaining erythrocytes were washed three times with 0·9% NaCl and diluted first to the volume of the original blood and then five times with water (Augustinsson, 1957).

From human serum. A purified preparation, Cohn's fraction IV-6 (Kabi AB, Stockholm), containing butyrylcholinesterase was used, 100 mg. being dissolved in 300 ml. of buffer *C*.

Substrates and inhibitors

Substrates. The following substrates were used: acetylcholine iodide, propionylcholine iodide, acetyl-β-methylcholine iodide and butyrylcholine iodide, all prepared according to Tammelin (1957*a*); triacetin (Hopkin and Williams Ltd.).

The final concentrations in the reaction vessels are given in the Figures. In the inhibitor experiments acetylcholine iodide was the substrate at p*S* 2·2.

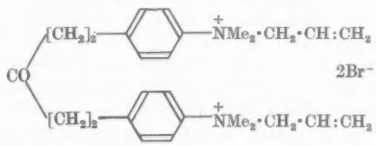
Table 1. *Highest and lowest values for the spontaneous hydrolysis of the different substrates used with the different inactivated enzyme preparations*

p*S* = $-\log$ (molar concn. of substrate).

		Spontaneous hydrolysis (μm-moles/ml./min.) at				
Substrate	Source of enzyme	p*S* 1·3	p*S* 1·6	p*S* 1·8	p*S* 2·2	p*S* 2·8
Acetylcholine	Goldfish	10·0	8·0	6·0	5·0	3·0
Butyrylcholine	Erythrocytes	2·0	1·5	1·0	0·5	0·5

Inhibitors. The following inhibitors were used: eserine (physostigmine salicylate, T. and H. Smith Ltd., London; Sarin (*iso*propyl methylphosphonofluoridate); B & W 284 C51 (I) mipafox (*NN'*-di*iso*propylphosphorodiamidic fluoride); DFP (di*iso*propylphosphorofluoridate); all these were prepared according to L.-E. Tammelin & L. G. Fagerlind (unpublished work); MEPtCh (Tammelin, 1957*b*:

$$Me{\cdot}PO(OEt){\cdot}S{\cdot}CH_2{\cdot}CH_2{\cdot}\overset{+}{N}Me_3I^-$$



B & W 284 C51 (I)

The final concentrations in the reaction vessels before adding the substrate are given. The times of incubation with the inhibitors were chosen so that maximum inhibition was obtained.

Composition of reaction mixtures. The reaction mixture before adding the substrate consisted of equal volumes of one of the buffers mentioned above containing the enzyme preparation, and water or inhibitor solution. The volume of the substrate solution added was 10% of the volume of this reaction mixture.

RESULTS

In Figs. 1–4 the results of the experiments on substrate specificities are shown. These experiments were performed on acetylcholinesterase preparations from goldfish muscle, electric organ of electric ray and erythrocytes and on a purified butyrylcholinesterase preparation from human serum.

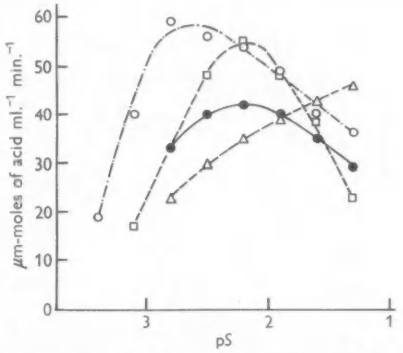


Fig. 1. Activity–p*S* curves for the enzymic hydrolysis of acetylcholine by the following cholinesterase preparations: goldfish muscles (●); electric organ of *Torpedo ocellata* (○); erythrocytes (□) (acetylcholinesterases); human serum (purified) (△) (butyrylcholinesterase).

In Fig. 1 are shown the results with acetylcholine. All the acetylcholinesterase preparations give the well-known 'bell-shaped' activity–p*S* curves. The maximum activities are obtained at slightly different values of p*S*. In the region of substrate concentration investigated the p*S* curve obtained with the butyrylcholinesterase preparation from serum conforms with the well-known 'S-shaped' curve (e.g. Augustinsson, 1949). It is observed that a maximum activity of about 20 μmoles of acetylcholine hydrolysed/mg. of dry

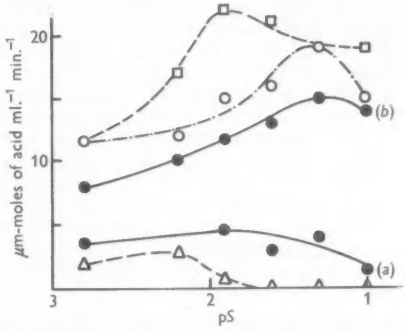


Fig. 2. Activity–p*S* curves for the enzymic hydrolysis of acetyl-β-methylcholine. (For explanation of symbols see Fig. 1.) Curve (*b*) was obtained by using twice the enzyme concentration used for (*a*).

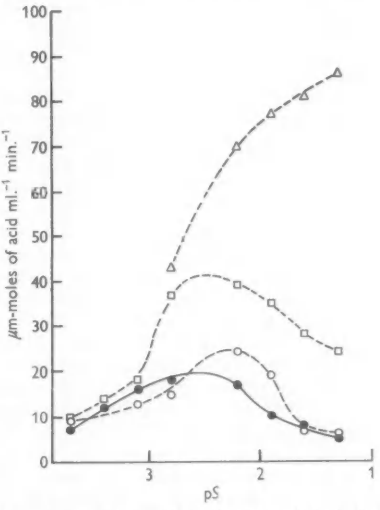


Fig. 3. Activity–p*S* curves for the enzymic hydrolysis of propionylcholine. (For explanation of symbols see Fig. 1.)

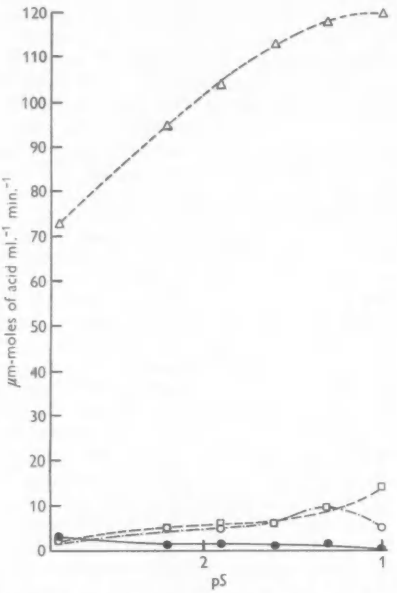


Fig. 4. Activity–p*S* curves for the enzymic hydrolysis of butyrylcholine. (For explanation of symbols see Fig. 1.)

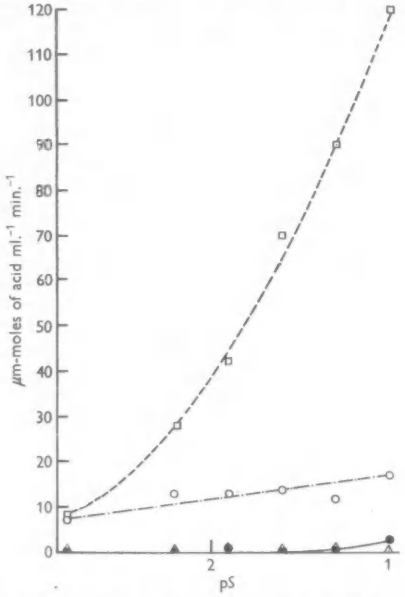


Fig. 5. Activity–p*S* curves for the enzymic hydrolysis of triacetin. (For explanation of symbols see Fig. 1.)

preparation/hr. is obtained with this preparation. This is in fairly good agreement with the value of 90 μmoles/mg./hr. obtained by Surgenor & Ellis (1954) with the same serum fraction (Cohn IV-6), particularly as the present preparation may have a reduced activity due to storage.

Acetylcholinesterase from goldfish has a very low activity with acetyl-β-methylcholine ((*a*) in Fig. 2). If the enzyme concentration is doubled, however, demonstrable activity with this substrate is present ((*b*) in Fig. 2). The butyrylcholinesterase activity with this substrate was very low.

The same general results have been obtained for propionylcholine (Fig. 3) as for acetylcholine although the relatively much higher activity of the butyrylcholinesterase preparation is to be noticed. This preparation has a high activity with butyrylcholine (Fig. 4). The acetylcholinesterase preparations from erythrocytes and electric ray have traces of activity with butyrylcholine, but that from goldfish has virtually none. Triacetin (Fig. 5) is rapidly hydrolysed by the erythrocyte preparation, slowly by the ray preparation, and very slowly by the goldfish and the serum preparations. As to the effect of the inhibitors on the three different acetylcholinesterase preparations the following results were obtained. The two non-selective inhibitors Sarin and eserine inhibit the enzymic activities of the three preparations within the same characteristic ranges of concentrations (Fig. 6). In Figs. 7 and 8 the values obtained with four selective

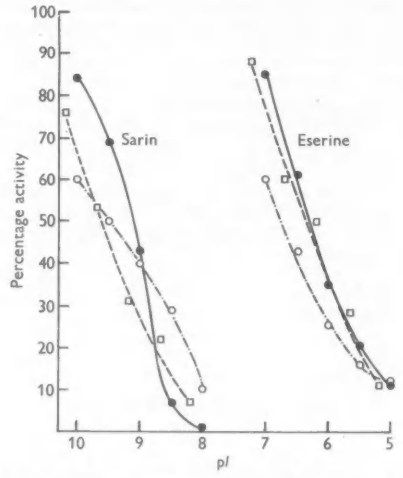


Fig. 6. Inhibiting action of eserine and Sarin on the following acetylcholinesterase preparations: goldfish muscles (●); electric organ of *Torpedo ocellata* (○); erythrocytes (□). Substrate, acetylcholine.

inhibitors have been plotted: B & W 284C51 and MEPtCh are known to be selective for acetylcholinesterase (Austin & Berry, 1953; Tammelin, 1958*b*), and mipafox and DFP for butyrylcholinesterase (Aldridge, 1953).

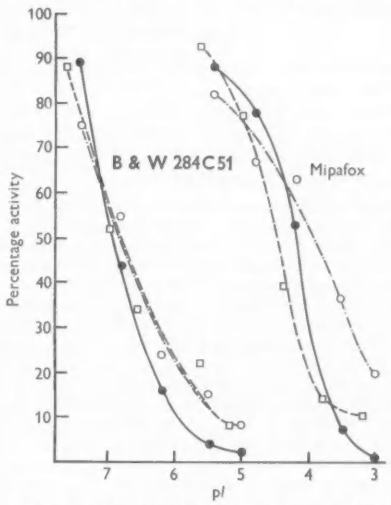


Fig. 7. Inhibiting action of B & W 284 C51 and mipafox on acetylcholinesterase preparations (see legend of Fig. 6). Substrate, acetylcholine.

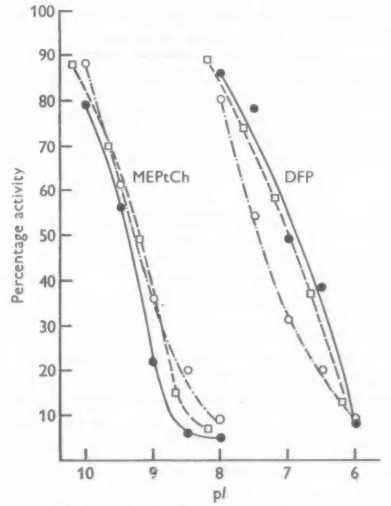


Fig. 8. Inhibiting action of MEPtCh and DFP on acetylcholinesterase preparations (see legend of Fig. 6). Substrate, acetylcholine.

The two selective acetylcholinesterase inhibitors give similar curves with all the three enzyme preparations. Both of the inhibitors selective for butyrylcholinesterase also inhibit all three acetylcholinesterase preparations at about the same concentrations. The effective concentration ranges for the acetylcholinesterase and the butyrylcholinesterase inhibitors are different.

DISCUSSION

The reason for choosing two different modifications of Michel's (1949) buffer for the determinations of the enzymic activities is that it was considered an advantage to use the standard methods for erythrocyte and serum cholinesterase already available in this laboratory. The only difference between the buffers of importance for this work is their different buffer capacity. As pointed out under Methods and Materials it is necessary to correct the activity values obtained for the spontaneous hydrolysis of the substrates. This hydrolysis is certainly rapid at the higher substrate concentrations and may cause considerable errors if disregarded (see Table 1). Hydrolysis measured at the lower concentrations, in the presence of inactivated enzyme, though small, was still too high to be accounted for by spontaneous hydrolysis (e.g. Tammelin, 1958*a*). Since at the time a plausible explanation for this was lacking it was considered better to use blanks with inactivated (heated to 100°) enzymes for every substrate and enzyme preparation instead of calculating the appropriate values.

One aim of this investigation was to confirm the classification of the cholinesterase activity of body muscles of goldfish as an acetylcholinesterase and to compare it with the acetylcholinesterase preparations from some well-known sources—erythrocytes and electric organ. The following relationships, as given among others by Augustinsson (1957), were considered suitable for investigation: (1) the form of the substrate concentration–activity curves; (2) the influence of different inhibitors, both reversible and irreversible, selective and unselective.

The results obtained with the different substrates make it clear that the goldfish preparation shows properties very similar to those obtained by the two other recognized sources of acetylcholinesterase. The preparation differs from the one containing butyrylcholinesterase. It is clear that when butyrylcholine, which may be regarded as a specific substrate for butyrylcholinesterase, is used little or no activity is obtained with the acetylcholinesterase preparations. The optimum substrate concentrations for the three preparations differ according to the source of enzyme and the substrate. This is well known (e.g. Augustinsson, 1949),

and does not modify the conclusion that acetylcholinesterase is present.

Of those tested here the only preparation splitting triacetin vigorously was that of the erythrocytes. This was to be expected, since acetylcholinesterase from erythrocytes does hydrolyse triacetin; furthermore, the preparation was not free from ali-esterases (Whittaker, 1951). Augustinsson (1949) has obtained a considerable activity with this substrate in preparations from both electric organ of electric eel (*Electrophorus electricus*) and muscles of guppy (*Lebistes reticulatus*). Acetylcholinesterase preparations from different fishes thus seem to differ in this respect.

An esterase is considered to be a cholinesterase if it is inhibited by 10^{-5}M-physostigmine (Augustinsson, 1957). This is true for the acetylcholine-hydrolysing activity of the goldfish preparation, as well as for those of electric ray and erythrocytes (Fig. 6). B & W 284C51 has been used as a selective inhibitor of acetylcholinesterase by several authors (e.g. Austin & Berry, 1953; Holmstedt, 1957). Recently, Tammelin (1958*b*) has shown methylethoxyphosphorylthiocholine to be a selective inhibitor of acetylcholinesterase. On the other hand, mipafox and DFP are known to be selective inhibitors of butyrylcholinesterase, their pI_{50} values [$-\log$ (concn. of inhibitor)] being between 7 and 8 (Holmstedt, 1957).

The results given in Figs. 7 and 8 show conformity between the three preparations tested. This is particularly clear for the two selective inhibitors of acetylcholinesterase. Also when treated with the selective inhibitors of butyrylcholinesterase the three preparations behave rather similarly. None is inhibited at significantly lower concentrations of the inhibitors than any of the others, the pI_{50} values being about 4 (mipafox) and 7 (DFP), instead of 7 and 8 as for butyrylcholinesterase. It thus seems reasonably well established that the extract from body muscles of goldfish contained acetylcholinesterase activity. Its substrate and inhibitor relations agree with those of the recognized acetylcholinesterase preparations from the electric organ of the electric ray and from erythrocytes.

SUMMARY

An extract from body muscles of goldfish (*Carassius auratus*) has been shown to contain acetylcholinesterase with the same substrate and inhibitor relationships as acetylcholinesterase preparations from human erythrocytes and from electric organ of electric ray (*Torpedo ocellata*).

My sincere thanks are due to the chief of this Institute, Professor G. Ljunggren, for his kind interest in my work, and to Professor P. E. Lindahl, Institute of Zoophysiology, University of Uppsala, for his valuable criticism. The skilful technical assistance of Mrs Kerstin Sjöström and Miss Ingegerd Brunnhagen is gratefully acknowledged.

REFERENCES

Aldridge, W. J. (1953). *Biochem. J.* **53**, 62.
Augustinsson, K.-B. (1949). *Arch. Biochem.* **23**, 111.
Augustinsson, K.-B. (1957). In *Methods of Biochemical Analysis*, vol. 5. Ed. by Glick, G. New York: Interscience Publ.
Austin, L. & Berry, W. K. (1953). *Biochem. J.* **54**, 695.
Holmstedt, B. (1957). *Acta physiol. scand.* **40**, 322.
Lundin, S. J. (1958). *Experientia*, **14**, 131.
Michel, H. O. (1949). *J. Lab. clin. Med.* **34**, 1564.
Surgenor, D. M. & Ellis, D. (1954). *J. Amer. chem. Soc.* **76**, 6049.
Tammelin, L.-E. (1953). *Scand. J. clin. & Lab. Invest.* **5**, 267.
Tammelin, L.-E. (1957*a*). *Acta chem. scand.* **11**, 487.
Tammelin, L.-E. (1957*b*). *Acta chem. scand.* **11**, 1340.
Tammelin, L.-E. (1958*a*). *Svensk kem. Tidsk.* **70**, 157.
Tammelin, L.-E. (1958*b*). *Ark. Kemi*, **12**, 287.
Whittaker, V. P. (1951). *Physiol. Rev.* **31**, 312.

An Unusual Property of Thyroxine and Other Iodophenols

By J. R. TATA*
National Institute for Medical Research, Mill Hill, London, N.W. 7

(*Received* 25 *August* 1958)

In the course of the work on enzymic deiodination of thyroid hormones, L-thyroxine and 3:5:3′-triiodo-L-thyronine, it was necessary to determine whether, and if so to what extent, spontaneous deiodination might occur. In some of the preliminary control experiments, which consisted of incubating thyroxine in enzyme-free aqueous buffer solutions, a surprising observation was made: when a solution of ^{131}I-labelled thyroxine in an aqueous organic solvent was diluted with a relatively large volume of aqueous buffer, there occurred virtually instantaneously a loss of radioactivity originally present in thyroxine. But later, mild shaking of the solution, without any other treatment whatsoever, produced a gradual reappearance of the lost radioactivity as thyroxine.

* Beit Memorial Fellow.

This unusual and hitherto undescribed property of thyroxine was thought of interest and the phenomenon was further studied with thyroxine and other related iodophenols. In this paper the influence of various conditions, many identical with those used for studying the deiodinating enzyme (Tata, Rall & Rawson, 1957), on the reversible disappearance of radioactive thyroxine and related iodophenols, is described.

EXPERIMENTAL

The properties of the following iodinated phenols were studied after labelling them with ^{131}I: L-thyroxine, 3:5:3′-tri-iodo-L-thyronine, 3:5-di-iodo-L-tyrosine, 3-iodo-L-tyrosine, 3-iodo-5-bromo-L-tyrosine and 3:5-di-iodo-*p*-cresol. The compounds were labelled in the iodine atoms *ortho* to the phenolic group by substitution with radioiodine (see Taurog & Chaikoff, 1957) to give a specific radioactivity of 0·5–3·0 mc/μmole. Thyroxine and tri-iodothyronine of higher specific activities were labelled by an exchange reaction and were obtained from Abbott Laboratories, Oak Ridge, Tenn., U.S.A. At least 90% of the total radioactivity was present as the iodinated phenol in each sample. All radioactive compounds were either stored in the dry state at 0° or frozen in 20–50% (v/v) aqueous propane-1:2-diol or butan-1-ol solutions.

The various buffers used were prepared according to Gomori (1955) in demineralized water (doubly glass-distilled water was also suitable). In general, the technique consisted in rapidly introducing 0·02–0·06 ml. of a solution of the radioactive iodinated compound, dissolved in 15–50% (v/v) aqueous propane-1:2-diol or butan-1-ol, into 2·5–3·0 ml. of the buffer; the diluted solution was then gently shaken at constant temperature in the presence of air. The usual temperature was 37°, although the effect of variation of temperature was also studied. At intervals of 5 min. to several hours after the mixing, portions (0·005–0·02 ml.) of the diluted iodophenol solution were withdrawn by capillary tubes for paper-chromatographic or electrophoretic analysis. For shorter time-intervals up to 30 sec., the mixing was done in haematological pipettes from which samples could be directly delivered for chromatography. The source of light used throughout the work was provided by two 40 w Osram Natural (84–23) fluorescent tubes placed at 100–150 cm. from the reaction vessels. Diffused red light was used to determine the effect of light on the reaction. Polythene apparatus replaced glassware in experiments for studying the influence of glass.

^{131}I-labelled constituents present in each sample were separated by chromatography on paper and the distribution of radioactivity was measured by automatic scanning and recording of chromatogram strips. The results were expressed as percentage of total ^{131}I remaining in the iodophenol fraction at any given time, after correction for the contaminant radioactivity present in the original solution in organic solvent. Quantitative chromatographic analysis, as used here, entails an error of $\pm$5–10% and figures given in the results represent average values derived from 2–6 determinations. The usual chromatographic solvent used was butan-1-ol–dioxan (4:1, v/v) saturated with 2N-NH_3; other solvent systems used are described elsewhere (Tata *et al.* 1957). Paper-electrophoretic analysis at 30–40 v/cm. for 10–20 min. was performed on Whatman paper no. 3 MM, with the same buffer as that used for dilution of the compound.

The ultraviolet-absorption spectra of thyroxine and tri-iodothyronine were determined in 0·1 M-Na_2CO_3 or 0·05 M-NaOH in 1 or 4 cm. quartz cells in a Hilger Uvispek spectrophotometer. The apparent ionization constants of the phenolic hydroxyl groups of the iodinated phenols were determined by the spectrophotometric method of measuring the molar extinction at different H^+ ion concentrations, as described by Gemmill (1955).

In some experiments the stability of iodine atoms was studied spectrophotometrically simultaneously with chromatographic analysis. For this purpose, the reaction was carried out in the absorption cell of the spectrophotometer by introducing the ^{131}I-labelled compound present in propane-1:2-diol into the cell containing the buffer and measuring the change in u.v. absorption at the wavelength maxima as a function of time; this change was compared with the change in distribution of radioactivity in samples withdrawn at different time intervals.

RESULTS

Some typical recordings of the distribution of radioactivity in chromatograms, prepared at different time intervals after the addition of ^{131}I-labelled thyroxine in 20% propane-1:2-diol to tris

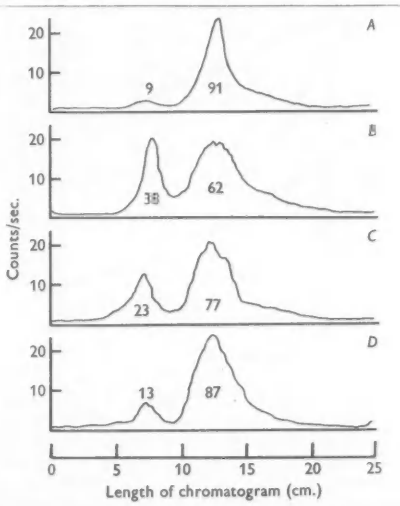


Fig. 1. Distribution of radioactivity on chromatograms of samples withdrawn at different time intervals after the mixing of 0·018 μg. of ^{131}I-labelled L-thyroxine dissolved in 0·05 ml. of 20% propane-1:2-diol with 2·5 ml. of tris-HCl buffer at pH 7·6. Time of withdrawal of samples: *A*, before mixing; *B*, 10 min., *C*, 180 min., *D*, 300 min., after mixing. Chromatographic solvent: butan-1-ol–dioxan–ammonia; the peak with low R_F resembles that of iodide, while the other peak represents thyroxine. The number within or above each peak is the percentage of radioactivity distributed in each peak.

(2-amino-2-hydroxymethylpropane-1:3-diol) buffer at pH 7·8, are shown in Fig. 1. At time zero, which shows the analysis of radioactive thyroxine as present in propane-1:2-diol before mixing with the aqueous medium, more than 90% of ^{131}I was present in the thyroxine fraction; 5 min. after the mixing, about 40% of the original thyroxine radioactivity had disappeared and was found at a position on the chromatogram similar to that occupied by iodide ion. It is shown below that the transient product formed was not iodide, as was suggested by its chromatographic properties. With the passage of time, as shown in Fig. 1, analysis of samples withdrawn from the same reaction vessel revealed that the lost radioactivity began to reappear spontaneously in the thyroxine fraction until almost the original pattern was obtained. During this period of time no intervention, except the withdrawal of small samples for chromatography, was involved. This phenomenon is not simply a dilution effect, because no change occurred when 0·02–0·06 ml. of the same thyroxine solution was introduced into 2·5 ml. of the original solvent (20% propane-1:2-diol).

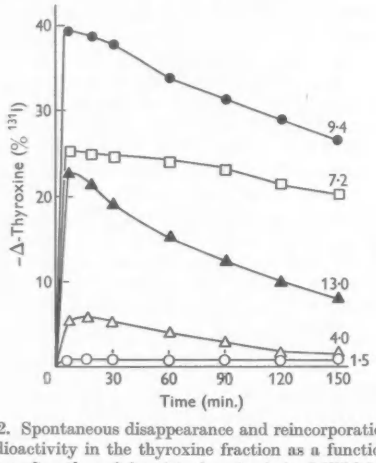


Fig. 2. Spontaneous disappearance and reincorporation of radioactivity in the thyroxine fraction as a function of time after the mixing (at time 0 min.) of ^{131}I-labelled thyroxine, dissolved in 18% propane-1:2-diol, with aqueous buffers of different pH values. Propanediol solutions (0·05 ml.) were added to 2·5 ml. of buffer and the final concentration of thyroxine was 10^{-7} M. The pH of the buffer is shown by the figure at the end of each curve. Buffers: pH 4·0, 0·05 M-citrate; pH 7·2, 0·05 M-tris-maleate–NaOH; pH 9·4, 0·05 M-Na_2CO_3–$NaHCO_3$; for pH 1·5 and 13·0, 0·08 N-HCl and 0·1 N-NaOH respectively were used. The term '−Δ-thyroxine' is used in this Figure (and in all subsequent Figures) to express the change in the percentage loss of ^{131}I in the thyroxine fraction in chromatograms of samples withdrawn after the mixing of the radioactive material.

The rate and extent of both the initial loss of radioactivity from thyroxine and its reappearance varied according to the pH of the aqueous medium. Fig. 2 depicts the influence of H^+-ion concentration on the reaction with L-thyroxine.

The first points represent the initial breakdown of radioactive thyroxine in buffers of different pH values on the basis of difference in thyroxine ^{131}I on chromatograms before (time 0) and after mixing. The initial reaction was very rapid; in some experiments the maximum loss of radioactivity had occurred 20 sec. after the mixing. Thereafter, the reaction had in effect reversed and the downward-sloping lines in Fig. 2 represent the reincorporation of radioactivity in the thyroxine fraction on further standing. The time taken for the reappearance of the original distribution of ^{131}I in the thyroxine fraction varied according to the pH of the aqueous dilution medium: about 100 min. at pH 3·0–4·0; 250–350 min. at pH 6·0–8·0 and 150–220 min. at pH 12·0–14·0. Both the forward and backward processes were influenced by the pH of the buffer, but to a different extent.

Qualitatively similar results were observed with other radio-iodinated compounds studied. Results showing the effect of pH on the initial loss of

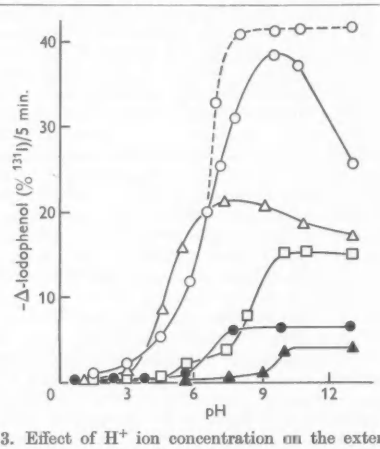


Fig. 3. Effect of H^+ ion concentration on the extent of disappearance of radioactivity, as seen chromatographically, from the various ^{131}I-labelled iodophenol fractions in the first 5 min. after the mixing has taken place. All substances were originally dissolved in 20% propane-1:2-diol, and 0·04 ml. of propanediol solution was added to 2·5 ml. of buffer. The nature of the buffer used had no effect. Final concentrations: 0·085 μM for thyroxine and tri-iodothyronine; 0·3 μM for other iodophenols. ○, L-Thyroxine; △, 3:5-di-iodo-*p*-cresol; □, 3:5:3′-tri-iodo-L-thyroxine; ●, 3:5-di-iodo-L-tyrosine; ▲, 3-iodo-L-tyrosine. The effect, with thyroxine, of replacement of all glassware by polythene apparatus is shown by the dashed line (○).

labelled iodine or the forward reaction are summarized in Fig. 3. The two major points of interest in the curves are: (1) Although identical conditions were employed for equimolar concentrations of the various iodophenols, the extent to which the initial breakdown occurred varied greatly from one compound to another. The following relationship for the magnitude of the reaction is obtained: thyroxine > di-iodo-*p*-cresol > tri-iodothyronine > di-iodotyrosine > monoiodotyrosine. This relationship is strictly valid for the forward reaction only and not for the reincorporation of radioactivity. The reaction with 5-bromo-3-iodotyrosine was too feeble to justify a comparison. (2) The optimum pH at which the very rapid loss of ^{131}I occurred was found to be different for the different iodophenols.

With all the *o*-iodophenols, the transient product formed occupied very nearly the same chromatographic position as that of iodide (R_F 0·25–0·30) in the butan-1-ol–dioxan–ammonia solvent system. Under similar conditions the R_F values of *o*-iodophenols here studied were as follows: L-thyroxine, 0·48; 3:5:3′-tri-iodo-L-thyronine, 0·64; 3:5-di-iodo-*p*-cresol, 0·89; 3:5-di-iodo-L-tyrosine, 0·10; 3-iodo-L-tyrosine, 0·19.

The dips observed in the pH curves at high pH values for thyroxine and di-iodo-*p*-cresol in Fig. 3 are caused by interference by some factor in glass produced under increasingly alkaline conditions. Thus, with thyroxine, the pH curve was found to attain a plateau, as shown by the dotted line, when polythene apparatus replaced all glassware.

Significance of the ionization of the phenolic group

In attempts to find the cause of this unusual property, the ionization of the phenolic hydroxyl group was implicated as being the most likely one, because this was the only common dissociable group in all the above compounds and the reaction occurred only during the passage of the iodophenol from the organic solvent into the aqueous medium. Results of the following two experiments supported the idea that the ionization of the phenolic hydroxyl group was indeed responsible for the self-reversible change in distribution of ^{131}I: (1) The pH at which the initial disappearance of the compound proceeded at half the maximal rate was found to correspond closely to the dissociation constant of the phenolic hydroxyl group of each iodophenol. Such a relationship is shown in Table 1. The values of pH for half maximal rate

Table 1. *Relationship between* pH-*dependence of forward reaction and the apparent* p*K values for the phenolic hydroxyl group of various iodophenols*

Compound	pH of ½ maximal initial ^{131}I disappearance	p*K* of phenolic OH (u.v. absorption)
L-Thyroxine	6·4	6·6
3:5:3′-Tri-iodo-L-thyronine	8·1	8·4
3:5-Di-iodo-*p*-cresol	4·9–5·8*	6·0
3:5-Di-iodo-L-tyrosine	6·6	6·5
3-Iodo-L-tyrosine	8·7	8·7

* It was not possible to reproduce values in a narrow range for this compound.

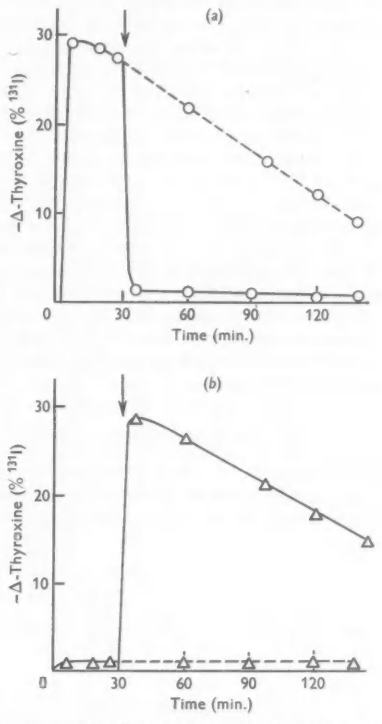


Fig. 4. Reversible effect of change in pH of the aqueous medium after the mixing has taken place. ^{131}I-Labelled thyroxine (0·54 μg.), dissolved in 0·06 ml. of 20% propanediol, was added to 2·5 ml. of 0·1 N-NaOH or 0·1 N-HCl, at time 0, and the loss or gain of radioactivity in thyroxine fraction determined chromatographically. (*a*) Aqueous medium was 0·1 N-NaOH. At 30 min. after mixing (shown by vertical arrow) enough N-HCl was added to lower the pH to 0·5. Subsequent analysis revealed rapid reappearance of all thyroxine radioactivity; control to which no acid was added is shown as the dashed line. (*b*) 0·1 N-HCl was the aqueous medium. At 30 min. after mixing (shown by vertical arrow) enough N-NaOH was added to raise the pH to 13·3. The change in pH is followed by instantaneous disappearance and slow reappearance of radioactive thyroxine. The dashed line indicates the control to which no alkali was added.

were derived from the pH curves in Fig. 3, while those of the apparent pK of the phenolic hydroxyl group were obtained by measurements of u.v.-absorption spectra of the different iodophenols. (2) The direct relationship between the degree of ionization of the phenolic hydroxyl group and the instability of the compounds was enhanced by the reversibility of both the forward and backward reactions brought about by a change in pH after the mixing of organic and aqueous media had taken place. Examples of two opposite effects obtained with thyroxine are illustrated in Fig. 4.

In Fig. 4*a*, it is seen that the suppression of the ionization of the phenolic-hydroxyl group caused by lowering the pH from 13·0 to 0·5 was accompanied by an instantaneous reappearance of the original amount of radiothyroxine. This effect was not due to the presence of salt formed by the neutralization of the alkali, because the addition of an equivalent amount of NaCl, without a diminution in the pH, had no effect. In Fig. 4*b*, it is seen that the sudden ionization of the phenolic group by making the medium alkaline was accompanied by the disappearance of thyroxine ^{131}I, followed by recombination in the usual manner. This feature of reversibility caused by change in pH was shared by all the other compounds.

Effect of other variables and light

The inclusion in the aqueous medium of organic solvents in which these iodophenols are highly soluble resulted in an inhibition of the initial reaction as exemplified in Table 2 for radiothyroxine. The extent of inhibition depended upon the organic solvent used and the final concentration at which it was present. It is possible that more than one phenomenon is involved in the inhibitory effect of the above solvents because the effect of dimethylformamide cannot be reconciled with that of other solvents, solely on the basis of a change in the dielectric constant of the medium.

The concentration of the iodophenol, temperature, the composition of the buffer and the ionic strength of the aqueous medium all had minor effects on the rate and extent of the reaction, which are not observed in enzymic deiodination. The variation in concentration of these iodinated compounds was limited, except at very high or low pH, by their extreme insolubility in water. However, in most cases, approximately the same fraction of radioactivity was found to disappear after the passage of the substance from organic to aqueous medium, over a wide range of concentration. But the concentration of the organic solvent in which the iodophenol was dissolved was important; in general, the extent of reaction occurring after mixing was proportional to the concentration of propane-1:2-diol or butan-1-ol. Variation of temperature over the range 7–57° had little effect. At most, a variation of 10° resulted in a few cases in only a 5–10% difference in reaction rates. Interchange of citrate, acetate, tris maleate, tris hydrochloride, phosphate and carbonate buffers at over-lapping pH values resulted in no change in the pH curves shown in Fig. 3.

Table 2. *Effect of inclusion of organic solvents in the aqueous buffer on the extent of initial* ^{131}I *disappearance*

Aqueous buffer: 0·05 M-tris, pH 7·8; original thyroxine solution in 25% propane-1:2-diol; final thyroxine concentration 0·095 μM.

Organic solvent	Final concn. (%, v/v)	$-\Delta$-L-Thyroxine (% ^{131}I/5 min.)	Inhibition (%)
None	—	48·3	—
Methanol	30	25·0	48·3
	70	3·6	92·5
Ethanol	30	21·7	55·0
	60	2·0	95·8
Propan-1-ol	30	24·0	50·4
	60	4·6	90·5
Butan-1-ol	10	28·5	41·0
	25	10·5	78·2
	40	3·0	93·8
Acetone	30	30·5	36·5
	70	2·8	94·2
Propane-1:2-diol	10	24·0	50·4
	20	8·6	82·2
	30	0·0	100·0
NN'-Dimethylformamide	10	30·0	36·8
	20	16·5	65·8
	30	4·5	90·7

In contrast with the slight effects of the above variables, the reaction was found to be extremely photosensitive. This photosensitivity is illustrated in Fig. 5, for thyroxine.

In diffused red light, the reaction of spontaneous ^{131}I loss was either abolished or drastically reduced in magnitude. In Fig. 5 it is further seen that when the same samples studied in the dark for 1·5 hr. after mixing were then exposed to light (of the same intensity as that used in previous experiments), an instantaneous disappearance and re-formation of radioactive thyroxine was produced. The extent of both the forward and backward reactions was dependent upon the pH in the same manner as in previous experiments.

Actual disappearance and re-formation of thyroxine and the nature of products formed

Before an attempt was made to determine the nature of the radioactive products formed it was necessary to ascertain whether the loss and gain of radio-iodine of the iodophenolic fraction, as judged by the radioactivity of the latter, represented its true disappearance and re-formation. For this purpose it was decided to measure simultaneously the changes in the ultraviolet-absorption spectrum of thyroxine and chromatographic distribution of radioactivity by performing the experiment directly in the quartz optical cell of a spectrophotometer. Unfortunately, at the only absorption maximum, at 325 mμ, so far described for thyroxine (Lemmon, Tarpey & Scott, 1950; Lissitzky, 1952; Gemmill, 1955; Lardy, 1955), the extinction was too low to be measured with accuracy at the low concentrations of thyroxine (10^{-8} to 10^{-5} M) used in this work, in spite of a linear relationship between concentration and extinction. However, on further examination it was found that thyroxine showed a second absorption maximum at 231 mμ and similarly tri-iodothyronine was found to exhibit absorption maxima at 227 and 318 mμ. The absorption spectra of thyroxine and tri-iodothyronine in 0·05 M-NaOH (performed 24 hr. after the solutions were prepared) are shown in Fig. 6.

For both compounds the extinction at shorter wavelengths was found to be higher at all concentrations than that at 318 or 325 mμ. A linear relationship between optical density and concentration, in the range 10^{-6} M to $1{\cdot}5 \times 10^{-5}$ M, was also obtained at the shorter wavelengths. Hence, it was possible to perform simultaneous chromatographic and spectrophotometric analyses after the ionization of the phenolic hydroxyl group. Results of one such experiment with thyroxine are summarized in Fig. 7.

Although the absorption at 231 mμ only is shown in Fig. 7, the full absorption spectrum between 210 and 350 mμ was measured at successive time intervals. It is seen in this figure that

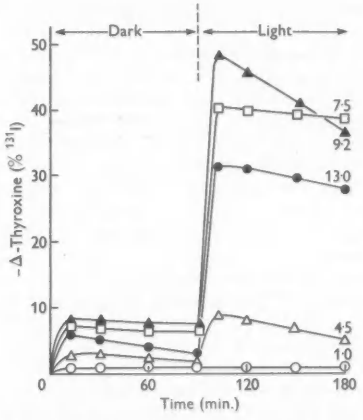


Fig. 5. Effect of light on the spontaneous disappearance and reappearance of radioactivity in the thyroxine fraction as determined chromatographically. ^{131}I-Labelled L-thyroxine (0·21 μg.) in 0·05 ml. of 20% propane-1:2-diol was mixed with 2·5 ml. of aqueous buffers of different pH values at time 0 min. in 'dark' (diffused red light). 92 min. after the mixing, light, of the usual intensity, was switched on and samples were chromatographically analysed for a further period of 90 min. Figures at the end of curves indicate the pH of the buffers of similar composition as shown in Fig. 2.

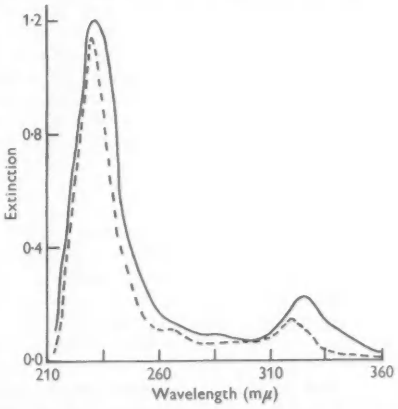


Fig. 6. Ultraviolet-absorption spectra, in a 1 cm. optical cell, of 30 μM-L-thyroxine and 3:5:3′-tri-iodo-L-thyronine dissolved in 0·05 N-NaOH. Absorption maxima: L-thyroxine, 231 and 325 mμ; 3:5:3′-tri-iodo-L-thyronine, 227 and 318 mμ.

the rate of increase in u.v. absorption at 231 mμ corresponds closely to the rate of re-formation of thyroxine as found by chromatography. These results strongly suggest that the disappearance and re-formation of thyroxine radioactivity in chromatograms reflects a true loss and gain of thyroxine.

However, the spectrophotometric studies shed no light on the nature of the transient radioactive product derived from thyroxine, and all attempts to solve this problem by other methods have been unsuccessful. From chromatographic analysis the only radioactive product formed had properties similar to those of iodide. But a consideration of the following results ruled out iodide as the transient radioactive product and showed that the deiodination, as observed chromatographically, was only an apparent one.

First, when both electrophoretic and chromatographic analyses were performed simultaneously on the same sample, a constant fraction of radioactivity appeared as iodide at all time intervals in electrophoresis diagrams; this fraction was equal to the percentage of ^{131}I present as iodide in the original organic solvent solution. The reaction product had the same electrophoretic behaviour as the parent iodophenol.

Secondly, only that fraction of ^{131}I present originally as iodide could be extracted from any sample as elementary iodine into CS_2 after acidification in the presence of Fe^{3+} ions or H_2O_2 and addition of ^{127}I as carrier. The acidification necessary for oxidation instantaneously caused the total reappearance of thyroxine or other iodophenol.

Thirdly, the presence of reducing agents such as thiosulphate, metabisulphite, dithionite and 2-thiouracil in the aqueous medium in concentrations up to 10^{-3} to 10^{-2} M (except for 2-thiouracil, because of low solubility) neither altered the kinetics nor prevented the reincorporation of ^{131}I into thyroxine or other compounds. Fortification of the buffer with increasing amounts of iodide also failed to alter the kinetics of the 'forward' and 'backward' processes.

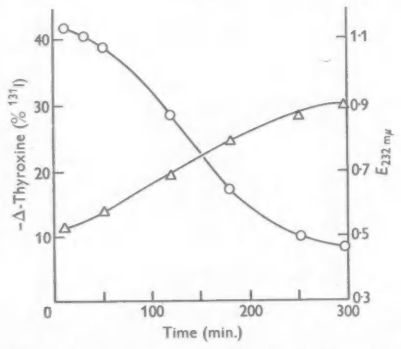


Fig. 7. Simultaneous chromatographic analysis (○) of radioactivity in thyroxine fraction and the change in ultraviolet absorption (△) as judged by the extinction 232 mμ (E): At time 0 min. ^{131}I-labelled thyroxine in 0·05 ml. of propane-1:2-diol was added to 2·7 ml. of 0·06 N-NaOH directly in a 1 cm. optical cell. Final concentration of thyroxine, 25 μM.

No partially de-iodinated radioactive products were formed from iodophenols labelled in more than one iodine atom, i.e. thyroxine, di-iodotyrosine and di-iodo-*p*-cresol. With di-iodotyrosine about 2–10 % of the total radioactivity appeared as thyroxine in chromatograms at the end of 1–4 hr. mixing with alkaline buffers at 37°.

The possibility of some kind of a reversible radioactivity-exchange reaction between iodide and the iodophenol was ruled out because, in spite of the addition of varying amounts of stable iodide or the organic solvent, the same fraction of ^{131}I appeared as the transformation product. Besides, the optimum alkaline pH values observed for the property here described are incompatible with the low pH and oxidative conditions essential for exchange reactions, already established for thyroxine, tri-iodothyronine and di-iodotyrosine (Miller, Anderson, Madison & Salley, 1944; Gleason, 1955; Taurog, 1955).

DISCUSSION

A transient disappearance and re-formation of ^{131}I-labelled thyroxine and structurally related iodophenols occurs when their solution in a small volume of organic solvent is diluted with a large volume of aqueous buffer. Although the general mechanism of this unusual reaction remains unknown, it is clear that the initial loss of labelled iodophenol is caused by the ionization of the phenolic hydroxyl group as a result of the passage from the organic to the aqueous medium. This conclusion has been drawn from the close relationship between the pH-dependence of the forward reaction and the pK values of phenolic-hydroxyl group for the different iodophenols and from the instantaneous reversibility of the reaction by changes in pH of the aqueous medium. As regards the pK values for phenolic hydroxyl groups, the figure of 8·4 reported here for 3:5:3′-tri-iodothyronine agrees better with that of 8·45 obtained by Gemmill (1955) than 9·2 reported by Lardy (1955).

The reaction as presented here might appear to be inconsistent with the second Law of Thermodynamics; this is probably due to the failure to detect an important step in the process and the identification of the transient product. None of the observations on the transient instability of iodophenols, conferred by the dissociation of the

phenolic hydroxyl group, was made under steady-state conditions. The initial velocity of the forward reaction is too rapid to be measured with the techniques available, being limited by the time taken for the preparation of chromatograms. The first sample withdrawn represented the point of maximum ^{131}I disappearance, followed by the re-formation of iodophenol and reciprocal diminution in the amount of reaction product.

One of the remarkable features of the reaction is its photosensitivity, demonstrated for thyroxine in Fig. 5. It is not known whether this photosensitivity is related to the long-known instability of alkaline solutions of thyroxine when exposed to light (Kendall & Osterberg, 1919). It must be pointed out that the intensity of light incident on the solutions in these experiments is quite high and much stronger than average daylight. The strong source of light was identical with that used for work on enzymic deiodination for which the present work was originally designed to provide control experiments.

All that can be said of the transient reaction product is that, in spite of chromatographic evidence, it is not iodide. In this respect, it is worth comparing the present reaction with the slow spontaneous deiodination observed in solutions of thyroxine and tri-iodothyronine (Wilkinson, Sprott, Bowden & Maclagan, 1954). In such a breakdown there is true formation of iodide as determined by electrophoresis and oxidation to elementary iodine; the distinction from the true (non-enzymic) de-iodination was best shown by analysing samples for about 6 days after the mixing of the organic solvent with the aqueous buffer. This is seen in Fig. 8 for a solution of thyroxine. The loss of radioactivity in the thyroxine fraction caused by the slow de-iodination is not reversible, either spontaneously or by acidification as for the transient reaction mediated by ionization of the phenolic hydroxyl group.

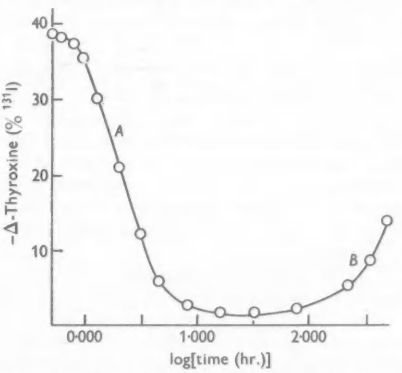


Fig. 8. Chromatographic determination of radioactivity in the thyroxine fraction over a long period of time (6 days) after the mixing. At time 0 min. 0·05 ml. of labelled thyroxine was added to 2·5 ml. of 0·05 M-tris HCl buffer at pH 9·5 (final concentration of thyroxine, 0·25 μM). Although the products formed looked chromatographically alike, in the part *A* of the curve no iodide was detected by other methods, whereas the irreversible formation of iodide in part *B* of the curve was due to a true deiodination.

It might seem that, by criteria of chromatographic analysis alone, the property here described could cause much confusion in interpreting results of work on enzymic de-iodination of thyroid hormones. (It is common practice to use thyroid hormones dissolved in propan-1:2-diol, butan-1-ol or methanol in studies of their metabolism by paper-chromatographic analysis, both *in vivo* and *in vitro*.) But a consideration of all the principal characteristics of this property, especially the spontaneous reversibility, the accelerated reversal by acids, the pH-dependence, the photosensitivity, independence of concentration, temperature and composition of the buffer and the lack of true iodide formation easily eliminate any doubt about its distinctness from all known biochemical reactions of thyroid hormones and related iodophenols. Further, in an extension of the present investigations it was found that the transient instability of thyroxine and tri-iodothyronine, caused by ionization of the phenolic-hydroxyl group, is most unlikely to occur in the presence of many proteins. Certain serum and tissue proteins, especially those that bind thyroid hormones, are capable of inhibiting the reaction and also of precipitating the reversal if they are added after the mixing of organic and aqueous media. Although enzymic de-iodination is inhibited by thyroxine-binding proteins the reaction cannot be reversed by them, once deiodination has taken place. This newer aspect of interaction between proteins and thyroid hormones is the subject of the next paper (Tata, 1959).

SUMMARY

1. When solutions of ^{131}I-labelled L-thyroxine and related iodophenols (3:5:3′-tri-iodo-L-thyronine, 3:5-di-iodo-L-tyrosine, 3-iodo-L-tyrosine and 3:5-di-iodo-*p*-cresol) in 15–50% aqueous organic solvents were introduced into a large volume of an aqueous buffer, there occurred a very rapid loss of a part of the radioactivity present in the iodophenol. This disappearance was followed by a gradual reincorporation of the radioactivity.

2. The rates of disappearance and re-formation of the radioactive iodophenol were determined by the pH of the aqueous medium and the presence of light. The concentration of the iodophenol, the

composition of the buffer and temperature had slight or no effect.

3. The initial reaction of the loss of iodophenol radioactivity was associated with ionization of the phenolic hydroxyl group in these compounds. The cause of the re-formation of the iodinated phenol was not discovered.

4. Although paper-chromatographic analysis indicated the formation of a radioactive substance of an R_F similar to that of iodide, during the initial reaction, results of other tests were incompatible with a mechanism of deiodination. With L-thyroxine, simultaneous chromatographic and spectrophotometric analysis showed a true increase in the concentration of the original substance during the reincorporation of ^{131}I into thyroxine.

5. The hitherto unreported ultraviolet-absorption maxima at 231 and 227 mμ for L-thyroxine and 3:5:3′-tri-iodo-L-thyronine respectively have been described.

I am indebted to Dr R. V. Pitt-Rivers, F.R.S., for help and advice throughout the investigations. I thank Dr W. L. Money, Sloan–Kettering Institute for Cancer Research, New York, N.Y., for providing me with labelled L-thyroxine and 3:5:3′-tri-iodo-L-thyronine of high specific radioactivity.

REFERENCES

Gemmill, C. L. (1955). *Arch. Biochem. Biophys.* **54**, 359.
Gleason, G. I. (1955). *J. biol. Chem.* **213**, 837.
Gomori, G. (1955). In *Methods in Enzymology*, vol. 1, p. 138. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Kendall, E. C. & Osterberg, A. E. (1919). *J. biol. Chem.* **40**, 265.
Lardy, H. (1955). *Brookhaven Symp. Biol.* no. 7, p. 90.
Lemmon, R. M., Tarpey, W. & Scott, K. G. (1950). *J. Amer. chem. Soc.* **72**, 758.
Lissitzky, S. F. (1952). Docteur-ès-Sciences Thesis: University of Paris.
Miller, W. H., Anderson, G. W., Madison, R. K. & Salley, D. J. (1944). *Science*, **100**, 340.
Tata, J. R. (1959). *Biochem. J.* **72**, 222.
Tata, J. R., Rall, J. E. & Rawson, R. W. (1957). *Endocrinology*, **60**, 83.
Taurog, A. (1955). *Brookhaven Symp. Biol.* no. 7, p. 111.
Taurog, A. & Chaikoff, I. L. (1957). In *Methods in Enzymology*, vol. 4, p. 856. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Wilkinson, J. H., Sprott, W. E., Bowden, C. H. & Maclagan, N. F. (1954). *Biochem. J.* **56**, 215.

A New Aspect of the Interaction Between Thyroxine and Proteins

By J. R. TATA*
National Institute for Medical Research, Mill Hill, London, N.W. 7

(*Received* 25 *August* 1958)

In the preceding paper details have been given about a hitherto undescribed property of thyroxine and some related iodophenols (Tata, 1959). This unusual property consists of a reversible loss of the ^{131}I-labelled iodophenol (as judged by its radioactivity) which occurs when the phenolic hydroxyl group undergoes ionization; such an effect is commonly observed during dilution of a solution of the ^{131}I-labelled compound in a small volume of organic solvent with a large volume of aqueous buffer. In view of the implication of such a reaction as a confusing factor in the study of enzymic deiodination of thyroid hormones, an examination of the temporary instability of the labelled iodophenol in the presence of biological material appeared desirable. The first results of such an examination revealed that the reversible loss of labelled thyroxine, as judged by its radioactivity, failed to occur if blood serum was present in the aqueous diluent. The spontaneous reaction was absent even when the serum was present at a fivefold dilution, and on further dilution the extent of inhibition was a function of the serum concentration. Serum behaved as an agent which masks the dissociation of the phenolic hydroxyl group of thyroxine in that it brought about an instantaneous re-formation of radioactive thyroxine if it was added to the aqueous medium after the initial loss of ^{131}I-labelled iodophenol had already taken place.

This effect of serum on the stability of thyroxine presented a new question: is it in any way related to the well-known interaction between certain serum proteins and thyroxine? Up to now the interaction has been studied as a selective binding of thyroxine to certain serum proteins as seen by co-precipitation (Taurog & Chaikoff, 1948), retention during dialysis (Trevorrow, 1939) and electrophoretic mobility (Gordon, Gross, O'Connor & Pitt-Rivers, 1952). Electrophoretic analysis has been most extensively used in characterizing serum 'thyroxine-binding protein' (TBP) and in determining the properties of binding of thyroxine by serum proteins (Robbins & Rall, 1957). Thus the

* Beit Memorial Fellow.

problem presented in the above question was attacked by a simultaneous electrophoretic study of binding of thyroxine by proteins and their action of 'stabilization' of thyroxine in the process of ionization of its phenolic hydroxyl group (the term 'stabilization' as used in this context refers only to the inhibition of the transient loss and re-formation of thyroxine and bears no reference to the long-term instability of thyroxine, observed on storage). In this way, the study of different human serum and rabbit tissue proteins revealed that the 'stabilization' effect of any fraction was indeed intimately related to its thyroxine-binding ability and the extent of inhibition of the lability of the iodophenol was a direct function of the concentration and 'affinity' of any given protein for binding thyroxine. The following is a detailed account of this new aspect of the interaction between proteins and thyroxine, and some practical applications of this effect in the biochemistry of thyroid hormones have also been proposed.

EXPERIMENTAL

The following proteins or protein mixtures were studied for their interaction with L-thyroxine or structurally related compounds: whole normal human serum, human serum albumin (Lister Institute), γ-globulin (Lister Institute), β-globulin (Fraction G-2, Lister Institute), β_1-globulin (Siderophilin, Protein Foundation Inc.), 'thyroxine-binding protein' in the form of Cohn fraction IV-6 of human serum (Freinkel, Dowling & Ingbar, 1955), human serum pre-albumin prepared by Schultze, Schönenberger & Schwick (1956) and rabbit skeletal muscle cellular thyroxine-binding protein fraction. The last fraction was prepared by a method similar to that described for rat skeletal muscle (Tata, 1958); the extract was then purified by electrophoresis on a cellulose column in 0·05M-tris (2-amino-2-hydroxymethylpropane-1:3-diol) buffer according to the method described by Flodin & Porath (1954) and the fraction of the eluate with maximal thyroxine-binding capacity was retained. Except for β_1-globulin (siderophilin), all protein samples were dissolved in the smallest volume of 0·05–0·1M-NaCl, dialysed against a large volume of 0·025M-NaCl and lyophilized. The freeze-dried preparations were used as such by weighing out the required amount for the individual reaction vessel. β_1-Globulin was dialysed against 0·05M-ethylenediaminetetra-acetate and stored as a 15% (w/v) stock solution.

L-Thyroxine, labelled with ^{131}I in the 3′ and 5′ positions, was stored, frozen in solution in 15% propane-1:2-diol. In some experiments, ^{131}I-labelled 3:5:3′-tri-iodo-L-thyronine, 3:5-di-iodo-L-tyrosine, 3-iodo-L-tyrosine and 3:5-di-iodo-*p*-cresol were also used. Radioactive thyroxine and tri-iodothyronine were obtained from Abbott Laboratories, Oak Ridge, Tenn., U.S.A.; the other substances were prepared in our laboratory by radio-iodination.

Buffers used were prepared according to Gomori (1955), except for those of extremely high or low pH, for which appropriate dilutions of HCl and NaOH were used. When proteins were added, the pH of the medium was measured again after the addition of proteins. Water dimineralized by ion-exchange was used throughout the work.

The techniques of observing and measuring the loss and reincorporation of ^{131}I in thyroxine or other iodophenol and the material used were identical with those described in the preceding paper (Tata, 1959). The temperature in all experiments was 37°. The binding of thyroxine to proteins was measured by electrophoresis on paper and in some cases supplemented by the measurement of ^{131}I in the protein precipitate obtained with 12·5% trichloroacetic acid. Electrophoresis was carried out either in 0·075M-veronal buffer (Block, Durrum & Zweig, 1955) or in the same buffer used for the incubation. The fraction of total radiothyroxine migrating with the protein fraction studied was determined by measuring the areas of distribution of ^{131}I on the electrophoresis diagram. The fraction of radioactivity that remained immobile on electrophoresis was judged to be the fraction of unbound or 'free' thyroxine.

A deiodinase from rabbit skeletal muscle was used to illustrate the effect of serum proteins on the enzymic deiodination of thyroxine. The skeletal muscle enzyme was obtained from an extract of low ionic strength (I, 0·02) by fractionation with 48–56% saturated $[NH_4]_2SO_4$, followed by heat treatment (43° for 2·5 min.) and adsorption on calcium phosphate gel (Tata, unpublished work).

RESULTS

Fig. 1 illustrates the effect of inclusion of serum proteins in the aqueous medium into which is introduced a small volume of ^{131}I-labelled L-thyroxine, dissolved in 20% propane-1:2-diol. In the protein-free buffer of pH 7·5, about 30% of

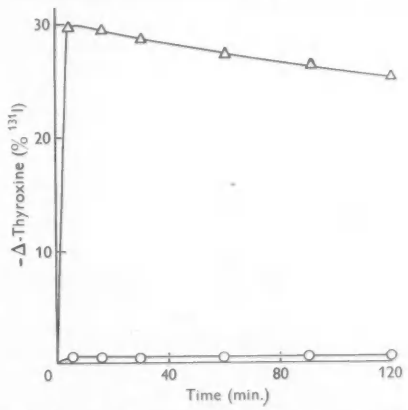


Fig. 1. Inhibitory effect of the presence of human serum in the aqueous buffer on the spontaneous disappearance of a part of radioactivity in the thyroxine fraction, as judged by chromatographic analysis. At time 0 min., 0·05 μg. of ^{131}I-labelled L-thyroxine in 0·04 ml. of 20% propane-1:2-diol was added to 2·8 ml. of the aqueous medium. ○, Buffer (tris maleate, pH 7·5) + human serum (diluted 1:4); △, buffer alone.

radioactivity in the thyroxine fraction had disappeared instantaneously after mixing, followed by a slow re-formation of radioactive thyroxine. But thyroxine failed to exhibit this property in the presence of human serum proteins at the same pH. In this particular case, the final concentration of serum proteins corresponded to a 1:4 dilution of whole serum. A quantitative relationship was observed between the final concentration of serum proteins and the extent to which the reaction was inhibited and this relationship is discussed below.

A similar 'stabilization' of thyroxine in the presence of serum proteins was observed at higher but not at lower pH values. A summary of the effect of total human serum proteins on the spontaneous loss of radiothyroxine in the first 5 min. is presented in Fig. 2. It was found that the addition of serum proteins partially or totally failed to suppress the initial transient loss of ^{131}I from thyroxine at pH values lower than 6·0. Although the reaction of spontaneous ^{131}I disappearance is less intense between pH 3·0 and 6·0 than in more alkaline media, the hump seen in the lower curve in Fig. 2 is very significant. The absence of the serum effect was most marked at pH 4·5, which is in the neighbourhood of the isoelectric point of many serum proteins.

Addition of serum proteins to a solution of labelled thyroxine 30 min. after dilution, to give the equivalent of serum diluted 1:4, caused the immediate reappearance of all the ^{131}I in thyroxine. A typical result is illustrated in Fig. 3. The final protein concentration determined the magnitude of the effect. An example of this is given in the second case in Fig. 3, in which only a partial immediate re-formation of thyroxine was produced when the final serum protein concentration corresponded to a 1:10 dilution. The remainder of ^{131}I then reappeared as thyroxine at the same slow rate seen in the control protein-free samples.

From these results it can be concluded that (*a*) the spontaneous and reversible breakdown of thyroxine cannot occur under physiological conditions in the presence of blood and (*b*) the net effect of serum is the same as that found under conditions that tend to suppress the ionization of the phenolic hydroxyl group of thyroxine (Tata, 1959).

Relationship between thyroxine-binding and 'stabilization' of thyroxine by proteins

In establishing this relationship, advantage was taken of the fact that the binding of thyroxine is localized in well-defined protein fractions of the

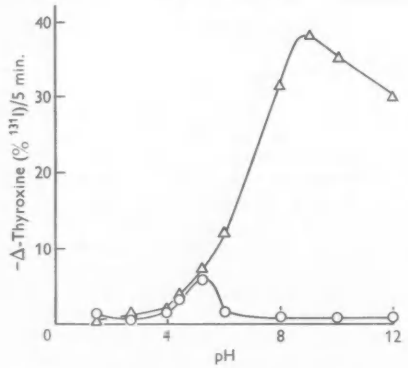


Fig. 2. Effect of H^+ ion concentration on the inhibition by human serum (diluted fourfold) of the spontaneous loss of radioactivity in the thyroxine fraction caused by mixing a solution of the labelled iodophenol in 20% propane-1:2-diol with aqueous buffers. The change in the radioactivity in the thyroxine fraction in chromatograms was recorded 5 min. after the mixing. Final concentration of thyroxine, 8–20 μmM. ○, Human serum + buffer; △, buffer alone. Buffers used: up to pH 2·5, acetic acid; pH 3·0–5·0, citrate; pH 5·0–7·0, phosphate; pH 7·0–9·0, tris maleate or tris HCl; pH 9·0–12·0, carbonate or NaOH.

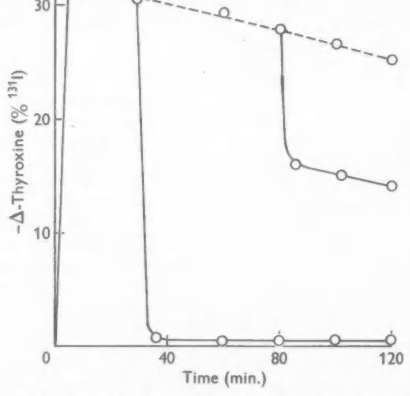


Fig. 3. Accelerated re-formation of radioactive thyroxine in chromatograms as a result of the addition of human serum after the initial disappearance of thyroxine ^{131}I had occurred in the usual manner. At the point indicated by vertical arrow *A*, sufficient human serum was added to obtain a final fourfold dilution; at the point of vertical arrow *B*, in another reaction vessel, the amount of human serum added gave a final 1:10 dilution. The dashed line represents the slow reappearance of thyroxine radioactivity in the serum-free control. Final concentration of ^{131}I-labelled L-thyroxine, 7 μmM; 0·04 ml. of thyroxine solution in 20% propanediol was mixed with 2·5 ml. of tris maleate buffer, pH 7·4.

serum; a comparison of the influence of thyroxine-binding and non-thyroxine-binding proteins on 'stabilization' of thyroxine hence proved useful. For this purpose, electrophoretic analysis was simultaneously performed on the same sample withdrawn for chromatographic determination of the distribution of ^{131}I.

Results obtained from such studies revealed that the thyroxine-binding property of proteins was indeed closely related to their 'stabilizing' action on thyroxine. This is illustrated in Fig. 4 for some human serum protein fractions. In the experiments illustrated, the final protein concentration in any fraction was adjusted to about 30% of what was estimated to be its concentration in normal human serum. It is thus seen that the extent to which thyroxine was 'stabilized' always very nearly corresponded to the fraction of thyroxine bound to the proteins. β- and γ-Globulin fractions which are feeble in binding thyroxine failed to affect significantly the spontaneous reaction; on the other hand, strong inhibition was exhibited by the 'thyroxine-binding protein' (Cohn fraction IV-6, in this case) and human serum albumin. Also the pre-albumin fraction, about which nothing was known at the time of this work as regards thyroxine-binding, showed the most intense stabilizing effect and a correspondingly intense thyroxine-binding power at concentrations much lower than for other proteins. More recently, Rich & Bearn (1958) and S. H. Ingbar (personal communication) have also found the pre-albumin fraction to be the most effective thyroxine-binding protein. Among the other protein fractions also tested in this manner, but not reported in Fig. 4, were human-serum β_1-globulin or metal-binding protein (siderophilin) and a preparation of cellular thyroxine-binding protein from rabbit skeletal muscle. The former had only a insignificant capacity for binding thyroxine or inhibiting the spontaneous disappearance of thyroxine, whereas the tissue extract occupied a position midway between human γ-globulin and serum albumin fractions.

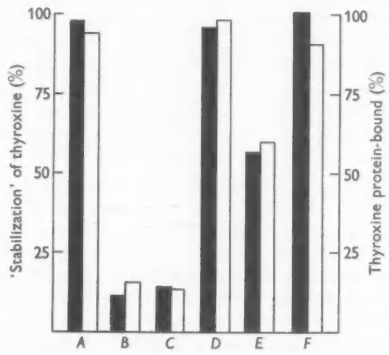


Fig. 4. Relationship between the 'stabilization' effect (black bars) and thyroxine-binding (open bars) by human serum and some of its protein fractions. The 'stabilization' effect was determined by measuring the percentage inhibition of initial loss of radioactivity, in the first 5 min. after mixing, in the thyroxine fraction (chromatographically) in the presence of proteins as compared to the protein-free buffer (0·05M-tris maleate, pH 7·5). The percentage of thyroxine protein-bound was determined by paper electrophoresis carried out on the same samples. Protein fractions and their concentrations: *A*, Whole human serum, 28·0 mg./ml.; *B*, γ-globulin, 5 mg./ml.; *C*, β-globulin (Lister fraction G-2), 5 mg./ml.; *D*, Cohn fraction IV-6, 5 mg./ml.; *E*, human serum albumin, 15 mg./ml.; *F*, pre-albumin, 2·5 mg./ml. Final concentration of thyroxine, 2·5 μmM. 0·04 ml. of propanediol containing thyroxine was mixed with 2·5 ml. of buffer.

If the pH of the medium and concentration of thyroxine were held constant, the only variable was the concentration of the protein. Thus the values for the percentage inhibition of ^{131}I disappearance and the fraction of thyroxine that was protein-bound both decreased to the same extent as the concentration of serum or protein fraction. The same was also true for the accelerated re-formation of thyroxine caused by the addition of thyroxine-binding proteins after the initial loss of ^{131}I had occurred. For example, in the experiment illustrated in Fig. 3, when samples were analysed for thyroxine-binding by electrophoresis, it was found that in the mixture to which the serum was added to obtain a final 1:4 dilution, over 90% of the thyroxine was bound to the protein. But in the sample containing the 1:10 dilution of serum, only about 50% of the thyroxine was bound to any protein, which corresponded to the extent of accelerated re-formation of thyroxine.

A more systematic study of the effect of protein concentration on the relationship between 'stabilization' of thyroxine and the protein-binding was then carried out with different protein fractions and some of the results are summarized in Fig. 5. All measurements were made using the same lowest possible levels of labelled thyroxine (0·003 μM) and the H^+ ion concentration was adjusted as near as possible to the physiological pH.

Besides providing further evidence for the identification of binding with stabilization, the results bring out the following characteristics: (1) As protein concentration fell, the values of inhibition of transient loss of thyroxine and the extent of binding to protein were found to fall off until both the functions were lost. Except at very low concentrations, the rate of fall was identical in both cases. Thus the extent to which the instability of thyroxine was inhibited by any binding protein

was directly proportional to the fraction of total thyroxine that was bound to the protein. (2) The rates at which the 'stabilization' effect and protein-binding diminished with decreasing concentrations of the proteins were different for the different proteins. (3) The minimal concentration of the protein fraction necessary to bring about binding or 'stabilization' depended on the protein studied. It was in increasing order as follows: human serum pre-albumin, Cohn fraction IV-6, human serum albumin, rabbit skeletal muscle thyroxine-binding protein fraction and γ-globulin (not shown in Fig. 5). (4) As seen by criteria of thyroxine-binding, studied by electrophoresis, or precipitation with trichloroacetic acid, and the inhibition of transient disappearance of thyroxine, human serum pre-albumin as isolated by Schultze *et al.* (1956) was found to exhibit the most intense interaction with thyroxine. This was valid both as regards the amount of protein required to obtain a given degree of binding or 'stabilization' and the narrow range of concentration observed for zero and maximum interaction.

At very low protein concentrations the issue is confused by competitive binding of thyroxine to the paper used for electrophoresis.

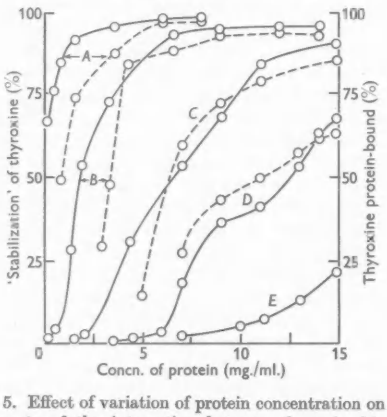


Fig. 5. Effect of variation of protein concentration on two aspects of the interaction between thyroxine-binding protein fractions and thyroxine, measured simultaneously. The 'stabilization' of thyroxine and the fraction of protein-bound thyroxine were determined as mentioned in Fig. 4, and all other experimental conditions were identical. O—O, Percentage of thyroxine 'stabilized'; O- - -O, percentage of thyroxine protein-bound; *A*, human serum pre-albumin; *B*, Cohn fraction IV-6; *C*, human serum albumin; *D*, rabbit skeletal muscle thyroxine-binding protein extract; *E*, human serum γ-globulin. It was not possible to determine the percentage of thyroxine bound to γ-globulin because of low electrophoretic mobilities of thyroxine and this protein fraction.

Interaction with tri-iodo-L-thyronine and iodinated tyrosines

In general, the same holds true for tri-iodo-L-thyronine as for L-thyroxine in the experiments described below, except that the extent of transient instability of tri-iodothyronine is small compared with that of thyroxine (Tata, 1959) which means that the values obtained for the 'stabilization' effect of proteins are less accurate. But all experiments with tri-iodothyronine confirm the previously described finding that the affinity of whole serum for this hormone is about one-third of that shown towards thyroxine (Robbins & Rall, 1955; Larson & Albright, 1955). The same is true for the 'stabilization' and binding effects with Cohn fraction IV-6 and serum pre-albumin, with which the lower affinity was even more marked. With the human serum-albumin fraction, however, the affinity for tri-iodothyronine was only slightly lower than for thyroxine.

With 3-iodo-L- and 3:5-di-iodo-L-tyrosines, although the transient instability of labelled iodophenol is very low, no significant 'stabilization' effect was detected for the different proteins in the concentrations of protein and iodinated compounds used for thyroxine and tri-iodothyronine. Not more than 10% of these amino acids were bound to any protein as seen by electrophoresis and less than 20% as seen by precipitation with trichloroacetic acid, which is in agreement with previous reports on the absence of any significant protein-binding of iodotyrosines (Tong, Taurog & Chaikoff, 1954; Robbins & Rall, 1957).

DISCUSSION

Whole human serum and some of its protein fractions and rabbit skeletal muscle protein(s) will interfere with the spontaneous and self-reversible loss of ^{131}I-labelled thyroxine, as judged by chromatographic analysis, during the ionization of the phenolic hydroxyl group, under conditions described in the preceding paper (Tata, 1959). The forward reaction (disappearance of radioactivity) is inhibited while the backward reaction (re-formation of radioactive thyroxine) is accelerated, hence conferring a 'stability' on thyroxine under conditions in which the phenolic hydroxyl group of the free compound would be partially or wholly ionized.

The mechanism and possible biological function of such an effect remain unknown, but it appears certain that the transient instability, or the loss and re-formation of thyroid hormones, cannot take place in the presence of most animal tissues and body fluids (no animal tissue or fluid has been shown to lack thyroxine-binding proteins). The

most intense interaction between proteins and the iodophenols, i.e. both the 'stabilization' and binding effects, were observed with the iodophenol which exhibits the highest degree of transient instability upon ionization of the phenolic hydroxyl group. Thus the lower degree of transient lability of tri-iodothyronine as compared with that of thyroxine (Tata, 1959) corresponds with the more feeble affinity of thyroxine-binding proteins for tri-iodothyronine. From this comparison it would appear that the degree of dissociation of the phenolic group determines the intensity of the interaction with proteins, as Robbins & Rall (1957) have suggested for the difference in binding affinity for the 3′:5′-dinitro and 3′:5′-dimethyl analogues of thyroxine. However, it is not only a question of the number of substituents *ortho* to the phenolic hydroxyl group or its p*K*, because there is even a more marked difference between thyroxine and diiodotyrosine. Such results also confirm the suggestions previously made (see Robbins & Rall, 1957) that all the major groups of the thyroxine molecule are essential for enabling it to combine more strongly with proteins than do other iodinated compounds.

Determination of thyroxine-binding power of proteins

While the mechanism of this interaction still remains unknown, this property has already been exploited usefully for solving some problems of biochemistry of thyroid hormones. These applications are based on the finding that the 'stabilization' of thyroxine is a direct function of the well-known binding of thyroxine by certain blood and tissue protein fractions. The most useful application so far has been that of studying the binding of thyroid hormones by proteins more quantitatively and under stricter physiological conditions than has been possible by other methods. If the percentage inhibition of the spontaneous loss of ^{131}I from thyroxine is measured at decreasing concentrations of a given protein fraction, then the lines obtained on plotting 'percentage of inhibition' against the 'logarithm of protein concentration' can be used for comparing the thyroxine-binding 'power' of different proteins. The slope of the line denotes the 'affinity' for binding thyroxine and the intercept that of the minimal protein concentration necessary for binding to occur at a fixed concentration of thyroxine. Alternatively, a comparison of the concentration of proteins necessary to obtain a 50% inhibition of the spontaneous loss of ^{131}I can also be used to evaluate the binding 'power' of a protein or protein fractions. The superiority of such a method over the conventional methods in which paper electrophoresis is used for measuring the interaction between proteins and thyroxine rests on the following advantages:

(1) It eliminates the unknown and variable factor of the binding of thyroxine by paper, used as a medium for electrophoresis. Interference by paper becomes substantial at low binding-protein concentrations and hence the non-electrophoretic method is particularly useful in studying thyroxine-binding by fluids of low protein concentration, such as tissue extracts and cerebrospinal and amniotic liquors.

(2) With the new method, only a trace amount of labelled thyroxine or related compound need be added and the relative protein mixture is not altered in comparing the binding potency of different proteins or protein mixtures. This is in contrast with electrophoretic analytical methods in which large amounts of thyroxine have to be added in order to observe the saturation point for a binding protein, or the introduction of different binding proteins in increasing amounts in order to obtain an effect of competition.

(3) With the method based on 'stabilization' of thyroxine, the interaction with proteins can be compared at physiological pH. Most workers have used alkaline media for electrophoretic analysis (see Robbins & Rall, 1957) or for equilibrium dialysis (Lein, 1952). A lower H^+-ion concentration is an important factor in the 'opening up' of binding groups of a protein which are not reactive under physiological conditions. This aspect is illustrated by comparing the 'stabilization' effects of proteins at different pH values, as shown in Fig. 6. Whereas almost the maximal 'stabilization'

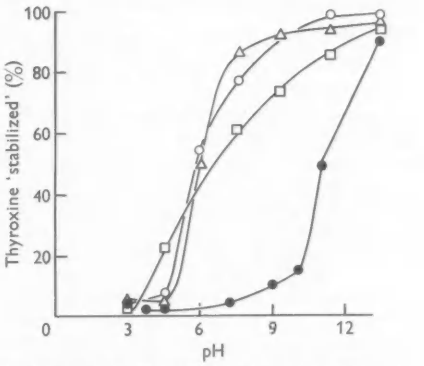


Fig. 6. Influence of pH on the 'stabilization' of thyroxine by human serum and some of its protein fractions. ○, Whole human serum (30 mg./ml.); △, Cohn fraction IV-6 (5 mg./ml.); □, human serum albumin (15 mg./ml.); ●, human serum γ-globulin (7 mg./ml.). The buffers used were the same as for those in Fig. 2; other conditions were identical with those for Fig. 4.

capacity was reached at pH 7·4 for whole serum or its major binding component, Cohn fraction IV-6, an increase in pH above 7·4 was accompanied by a further increase in 'stabilization' effect in the case of the albumin fraction. γ-Globulin, which normally does not bind or 'stabilize' thyroxine, was found to behave as binding proteins do, when the pH value was greater than 9·5. Later, it was confirmed that a similar situation also exists for thyroxine binding as measured by electrophoretic methods with buffers at varied pH.

(4) The method here proposed is useful in cases where electrophoretic separation of the possible binding components is doubtful. An example of this application has been the recent work on the absence in birds of significant thyroxine-binding by an α-globulin fraction (J. R. Tata & C. J. Shellabarger, unpublished work). In chick and duck sera most binding of endogenous and exogenous thyroid hormone was found to occur to albumin and there was no significant difference in the affinity of this fraction for thyroxine and tri-iodothyronine. This last finding can explain the identical biological activities of the two hormones in the chick (Shellabarger, 1955; Newcomer, 1957) and probably also the higher activity of tri-iodothyronine in mammals.

It should, however, be emphasized that the method based on the 'stabilization' of thyroxine is useful only for comparing the binding 'powers' of different proteins or protein mixtures and does not give any indication of the number of binding components in a mixture of proteins. For this purpose, it would be advantageous to use this method in conjunction with methods based on the electrophoretic separation of proteins.

Distinction between true deiodination and self-reversible loss of thyroxine, as judged by paper chromatography

A quite different principle has been used for the distinction between a true enzymic deiodination and the transient deiodination caused by ionization of the phenolic hydroxyl group when thyroxine or another iodophenol passes from an organic to aqueous medium and when the method of analysis is chromatographic. The principle is briefly illustrated in Fig. 7.

The conditions for the deiodinating enzyme were adjusted so that the initial velocity of the reaction approximately corresponded to the rate of loss of radioactive thyroxine in the spontaneous reaction. In both the enzymic and non-enzymic reactions the radioactive product appeared as 'iodide' in paper chromatograms. If human serum or any thyroxine-binding protein fraction was added a few minutes after the start of the enzymic reaction, further disappearance of thyroxine was inhibited and chromatographic analysis at subsequent time intervals showed no change in the amounts of thyroxine and iodide. Electrophoretic analysis revealed that the removal of the substrate by binding of thyroxine to serum proteins was responsible for the inhibition of the 'deiodinase'. However, when human serum was added to the reaction vessel containing the buffer alone, there was an instantaneous reappearance of the original ^{131}I in the thyroxine fraction and the disappearance of what appeared chromatographically as 'iodide', as in the experiments illustrated in Fig. 3. With purified 'deiodinase' solutions of low protein concentration, both reactions might occur simultaneously (based on paper-chromatographic analysis) but the difference in values of the iodide and thyroxine fractions before and after the addition of binding protein will then indicate the extent of true enzymic deiodination. This simple test for

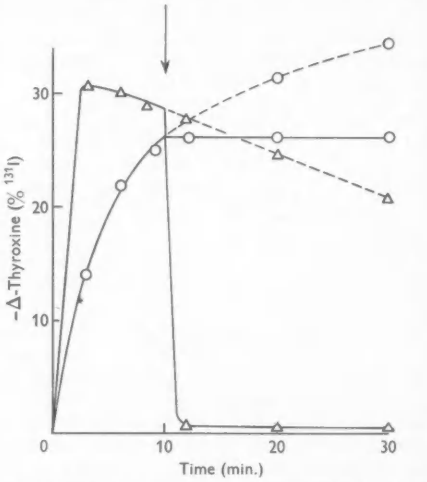


Fig. 7. Distinction between true enzymic deiodination (○—○, ○- - -○) and transient loss and re-formation of thyroxine (Δ—Δ, Δ- - -Δ) by addition of thyroxine-binding proteins, 10 min. (vertical arrow) after either reaction had started. Sufficient amount of whole serum, Cohn fraction IV-6 or serum albumin was added to bind all free thyroxine. The broken lines show how the two reactions would proceed if no protein were introduced. The transient reversible loss of thyroxine was produced by the mixing of 0·05 ml. of ^{131}I-labelled thyroxine solution in 20% propanediol with 2·5 ml. of tris maleate buffer at pH 7·5. In both this reaction and enzymic deiodination the radioactivity lost from thyroxine fraction appears as iodide in paper chromatograms. Enzyme was prepared in tris maleate buffer, pH 7·5. Final concentration of thyroxine added in both cases, 40 μmm.

differentiating between a true enzymic deiodination and an apparent, spontaneous deiodination has been routinely applied in our laboratory in studies on the purification and properties of the deiodinating enzyme.

The studies here presented raise the question of the importance of the ionization of the phenolic hydroxyl group in problems of thyroid-hormone biochemistry. For example, the function of binding or transport proteins in these studies appears to be the masking of the ionization of the phenolic group of thyroxine which normally, at a physiological pH, would be half-ionized (pK of the phenolic hydroxyl group of thyroxine, 6·4). The question is hence raised whether only the ionized thyroxine molecules are available for penetrating into tissue cells or for combining with some sites on the cell wall. A further question arises as to whether the degree of ionization of the phenolic group of thyroxine is related to the rate of interaction with enzymes controlling cellular oxidation or the control of permeability of membranes with respect to substrates of these enzymes.

SUMMARY

1. The transient instability of thyroxine and related iodophenols described previously (Tata, 1959) was inhibited by the presence of human serum in the aqueous medium, even when the serum was diluted fivefold. If the serum was added after the initial loss of thyroxine was noted, it caused the instantaneous reappearance of the original amount of thyroxine. Thus serum behaved as an agent masking the ionization of the phenolic hydroxyl group of the iodophenol.

2. The following human serum protein fractions were tested for the thyroxine 'stabilization' effect: albumin, γ-globulin, β-globulin, pre-albumin, Cohn fraction IV-6, and β_1-globulin. A rabbit skeletal muscle extract with thyroxine-binding properties was also tested. The effects were observed in decreasing order, with human serum pre-albumin, Cohn fraction IV-6, albumin and rabbit muscle extract; β-, γ- and β_1-globulins had very little, if any, influence on the stability of thyroxine.

3. By simultaneous electrophoretic analysis, it was found that the 'stabilization' effect of any protein was a direct function of the well-known thyroxine-binding properties. By studying the interaction with binding proteins at different dilutions, the fraction of thyroxine 'stabilized' was found to be equal to the protein-bound fraction. Human serum pre-albumin exhibited more intense binding and 'stabilization' properties than any other protein fraction.

4. Although the mechanism of this new aspect of interaction between proteins and thyroxine is unknown, the 'stabilization' effect has been applied in studying some problems of the biochemistry of thyroid hormones. The superiority of the 'stabilization' method over the conventional electrophoretic method for studying thyroxine-binding have been discussed. A new method has been proposed for distinguishing between a true and apparent deiodination of thyroid hormones, when studied by chromatographic analysis. These studies also point out the possible importance of the ionization of the phenolic hydroxyl group in some biochemical problems related to thyroid hormones.

I am indebted to Dr R. V. Pitt-Rivers, F.R.S., for valuable help and advice during the course of the work. I am also grateful to Dr W. d'A. Maycock, The Lister Institute of Preventive Medicine, Elstree, Herts, for generous supplies of human serum albumin and β- and γ-globulins; to Professor Dr H. E. Schultze, Behringwerke A.-G., Marburg/Lahn, for a sample of human serum pre-albumin; to Dr R. B. Pennell, Director, Protein Laboratories of Protein Foundation, Inc., Jamaica Plain, Mass., for β_1-globulin (siderophilin) and to Dr S. H. Ingbar for Cohn fraction IV-6. ^{131}I-Labelled L-thyroxine and 3:5:3′-tri-iodo-L-thyronine were kindly supplied by Dr W. L. Money, Sloan–Kettering Institute for Cancer Research, New York, N.Y.

REFERENCES

Block, R. J., Durrum, E. L. & Zweig, G. (1955). *A Manual of Paper Chromatography and Electrophoresis*. New York: Academic Press Inc.

Flodin, P. & Porath, J. (1954). *Biochim. biophys. Acta*, **28**, 91.

Freinkel, N., Dowling, J. T. & Ingbar, S. H. (1955). *J. clin. Invest.* **34**, 1698.

Gomori, G. (1955). In *Methods in Enzymology*, vol. 1, p. 138. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Gordon, A. H., Gross, J., O'Connor, D. & Pitt-Rivers, R. (1952). *Nature, Lond.*, **169**, 19.

Larson, F. C. & Albright, E. C. (1955). *Endocrinology*, **56**, 737.

Lein, A. (1952). *Fed. Proc.* **11**, 91.

Newcomer, W. S. (1957). *Amer. J. Physiol.* **190**, 413.

Rich, C. & Bearn, A. G. (1958). *Endocrinology*, **62**, 687.

Robbins, J. & Rall, J. E. (1955). *J. clin. Invest.* **34**, 1331.

Robbins, J. & Rall, J. E. (1957). *Recent Progr. Hormone Res.* **13**, 161.

Schultze, H. E., Schönenberger, M. & Schwick, H. (1956). *Biochem. Z.* **328**, 267.

Shellabarger, C. J. (1955). *Poult. Sci.* **34**, 1437.

Tata, J. R. (1958). *Biochim. biophys. Acta*, **28**, 91.

Tata, J. R. (1959). *Biochem. J.* **72**, 214.

Taurog, A. & Chaikoff, I. L. (1948). *J. biol. Chem.* **171**, 439.

Tong, W., Taurog, A. & Chaikoff, I. L. (1954). *J. biol. Chem.* **207**, 59.

Trevorrow, V. (1939). *J. biol. Chem.* **127**, 737.

The Translocation of Antibiotics in Higher Plants

3. THE ESTIMATION OF GRISEOFULVIN RELATIVES IN PLANT TISSUE*

By S. H. Crowdy, A. P. Green, John Frederick Grove, P. McCloskey and A. Morrison

Imperial Chemical Industries Ltd., Akers Research Laboratories, Welwyn, Herts

(*Received* 29 *September* 1958)

Crowdy, Gardner, Grove & Pramer (1955) reported on the recovery of griseofulvin [*dd*-7-chloro-4:6:2′-trimethoxy-6′-methylgris-2′-en-3:4′-dione, (II; R = OMe)] from treated broad-bean plants and described methods of estimating griseofulvin in the treating solution and in the plant tissue extracts. These methods were used by Crowdy, Grove, Hemming & Robinson (1956) to study the rates of uptake and decay of griseofulvin in plant tissue. These studies have now been extended to include eleven compounds closely related chemically to griseofulvin, and the present paper describes analytical methods for estimating these compounds in bean tissue extracts and in the treating solutions. With the exception of the acidic trione (II; R = OH: enolic form) and the basic amino derivative (II; R = NH_2) the compounds studied were neutral. Reasons underlying the selection of these compounds and discussion of the results obtained with them form the subject matter of the next paper (Crowdy, Grove & McCloskey, 1959).

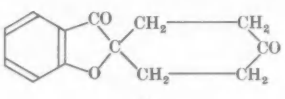
The analytical methods were essentially the same as those used for griseofulvin (Crowdy *et al.* 1955). Compounds remaining in the treating solution after a given period of uptake were estimated spectrophotometrically after solvent extraction and chromatography of the extract. Solvent extracts of the roots and shoots were subjected to countercurrent distribution and the compounds estimated spectrophotometrically after further purification by chromatography. Special methods of estimation were necessary for some compounds and these are described in detail.

The identification and estimation in bean tissue of metabolites of the griseofulvin relatives studied is also described, together with details of a search for griseofulvin metabolites formed in the decay experiments recorded by Crowdy *et al.* (1956).

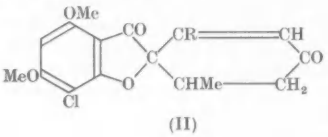
EXPERIMENTAL

Compounds. The compounds studied (Table 1) comprised the synthetic grisan-3:4′-dione (I) (McCloskey, 1958), the *ld*-diastereoisomer of griseofulvin (II; R = OMe) (MacMillan, 1959), the isomeric methyl enol ether (IV), and the dechloro analogue (VI; R = OMe) (MacMillan, 1953) of griseofulvin, three homologues of griseofulvin comprising

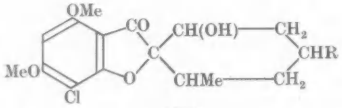
* Part 2: Crowdy, Grove, Hemming & Robinson (1956).



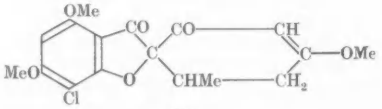
(I)



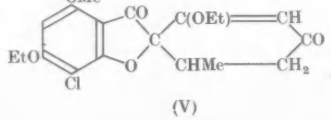
(II)



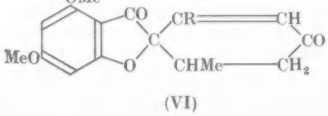
(III)



(IV)



(V)



(VI)

the *n*-propyl and *n*-butyl compounds (II; R = OPr^n and OBu^n respectively) (Duncanson, Grove & Jeffs, 1958) and the bisethoxy relative (V) (Duncanson, Grove, MacMillan & Mulholland, 1957), two neutral alcohols (III; R = H and OH), reduction products of the acidic trione (II; R = OH) (Grove, MacMillan, Mulholland & Rogers, 1952*a*), the trione (II; R = OH) and the basic amino compound (II; R = NH_2).

The melting point, 220°, of the alcohol (III; R = H) (cf. m.p. 198–200°, Grove *et al.* 1952*a*) is that of a dimorphic form obtained by crystallization from benzene–light petroleum (b.p. 60–80°).

MacMillan (1959) has shown that griseofulvin can be converted into a diastereoisomer in which the configuration at the spiran centre of asymmetry is inverted. The absolute configurations of these compounds have been deduced and can be expressed by using the convention of Cahn, Ingold & Prelog (1956). In this system correlation of configuration in a series of related compounds is often obscured; to overcome this difficulty in griseofulvin chemistry *d* and *l* are used to denote configuration at the asymmetric centres, the spiran centre being that first mentioned. Thus, griseofulvin is *dd*-7-chloro-4:6:2′-trimethoxy-6′-methylgris-2′-en-3:4′-dione and the above diastereoisomer the *ld* compound. With the exception of this compound all the derivatives of griseofulvin used in the present study have the *dd* configuration. For the grisan nomenclature see Grove, MacMillan, Mulholland & Rogers, 1952*b*).

Recovery techniques. All the compounds sublimed, unchanged, at 20–30° below the melting point at pressures between 10^{-1} and 10^{-6} mm.; recovery was not quantitative. The neutral compounds (and the amine II; R = NH_2) were all recovered quantitatively from an alumina column (pH 4: activated at 250° for 3 hr.) by elution with benzene–methanol mixtures. The acidic trione (II; R = OH) was recovered quantitatively from a Celite 545 (Johns–Manville Co. Ltd.) column buffered at pH 7·0 (Sørensen phosphate buffer) by elution with ether.

Griseofulvin was stable in aqueous solution at room temperature during 14 days, and hydrolysis to the trione (II; R = OH) was not appreciable: Griseofulvin (25 mg.) dissolved in acetone (5 ml.) was dispersed in water (1 l.). After standing with occasional stirring for 14 days at 25° (some crystallization took place) the solution was extracted with ether (4 × 300 ml.). The ethereal solution was concentrated to 200 ml. and extracted with 2 N-sodium hydroxide (2 × 45 ml.). The aqueous alkaline layer was re-extracted with ether (2 × 50 ml.) and made up to 100 ml. The ultraviolet-absorption of this solution (*l*, 1 cm.) showed only a very weak peak at 290 mμ (trione content <0·1 mg.).

Some of the griseofulvin relatives were more susceptible to hydrolysis, particularly during recovery from a large volume of wet solvent after countercurrent distribution. Thus small quantities of the acidic trione (II; R = OH) were frequently obtained after recovery of (IV) and the griseofulvin propyl and butyl homologues; and the trione (VI; R = OH) was obtained from the dechloro compound (VI; R = OMe). The two triones were produced by hydrolysis during working up after countercurrent distribution, since they normally appear in tubes well removed from the series containing the parent compounds. In these cases the estimates of the amount of the particular compound present were corrected by addition of weights equivalent to the triones produced.

Table 1. *Properties of griseofulvin relatives*

Compound	Structure	Formula	*M*	M.p.
ld-7-Chloro-4:6:2′-trimethoxy-6′-methylgris-2′-en-3:4′-dione	(II; R = OMe)	$C_{17}H_{17}O_6Cl$	352·8	220–221°
4:6:2′-Trimethoxy-6′-methylgris-2′-en-3:4′-dione	(VI; R = OMe)	$C_{17}H_{18}O_6$	318·3	179–181
7-Chloro-4:6:4′-trimethoxy-6′-methylgris-3′-en-3:2′-dione	(IV)	$C_{17}H_{17}O_6Cl$	352·8	200–201
7-Chloro-4:6-dimethoxy-6′-methyl-2′-*n*-propoxygris-2′-en-3:4′-dione	(II; R = OPr^n)	$C_{19}H_{21}O_6Cl$	380·8	153–154
2′-*n*-Butoxy-7-chloro-4:6-dimethoxy-6′-methylgris-2′-en-3:4′-dione	(II; R = OBu^n)	$C_{20}H_{23}O_6Cl$	394·8	156
7-Chloro-6:2′-diethoxy-4-methoxy-6′-methylgris-2′-en-3:4′-dione	(V)	$C_{19}H_{21}O_6Cl$	380·8	172
7-Chloro-4′:6′-dihydroxy-4:6-dimethoxy-2′-methylgrisan-3-one	(III; R = OH)	$C_{16}H_{19}O_6Cl$	342·8	222–224
7-Chloro-6′-hydroxy-4:6-dimethoxy-2′-methylgrisan-3-one	(III; R = H)	$C_{16}H_{19}O_5Cl$	326·8	220
7-Chloro-4:6-dimethoxy-2′-methylgrisan-3:4′:6′-trione	(II; R = OH)	$C_{16}H_{15}O_6Cl$	338·8	255–258 (decomp.)
2′-Amino-7-chloro-4:6-dimethoxy-6′-methylgris-2′-en-3:4′-dione	(II; R = NH_2)	$C_{16}H_{16}O_5NCl,H_2O$	355·8	258 (decomp.)
Grisan-3:4′-dione	(I)	$C_{13}H_{12}O_3$	216·2	89–90

Slight hydrolysis of griseofulvin homologues also took place during recovery from the treating solution, and this was allowed for by carrying out a control experiment similar to the one described above for griseofulvin. When the amount of trione isolated was significantly in excess of that found in the control, it was considered to be a metabolite (see p. 234).

Plants and preparation of plant extracts

Where two experiments were carried out with the same compound, the letters *A* and *B* are appended in Table 2.

The conditions under which the plants, *Vicia faba* L., var. Sutton's Dwarf, were raised, treated and harvested have been described (Crowdy *et al.* 1955). Roots and shoots were weighed immediately after harvesting and the weights recorded as the fresh weights. The pH of all treating and control solutions was initially $5{\cdot}6 \pm 0{\cdot}5$, and during the period of treatment rose to a final value of $7{\cdot}0 \pm 0{\cdot}5$ in the controls. In some cases (recorded in Table 2) the final pH of the treating solutions was outside this range.

With the propyl and butyl homologues of griseofulvin, 25 mg. of the compound was dissolved in a little acetone, and the solution was dispersed in 5 l. of water and supplied to 25 beakers each containing four plants: the solubility of the remaining compounds permitted the use of solutions having a concentration of 25 mg./l. (125 mg. in 5 l.), although this figure was sometimes considerably in excess of the solubility of the compound in pure water. With grisan-3:4′-dione one experiment (*B*) was carried out in which 400 mg. was dissolved in 16 l. of water and the solution supplied to 320 plants in 80 beakers.

The weight of water transpired during treatment was noted (Crowdy *et al.* 1956) and plants were harvested at the end of 3 days. The fresh weights of shoots and roots were determined separately and, with the exceptions detailed below, the tissues were then macerated in chloroform and extracted with the same solvent as previously described (Crowdy *et al.* 1955).

The trione (II; R = OH). In early experiments with the trione (II; R = OH) of which *A* (Table 2) is typical, roots and shoots were macerated in water and the acidified aqueous macerate was extracted with ether. In a later experiment (*B*) sodium hydrogen carbonate solution was used during maceration and the resulting solutions were acidified to Congo red with concentrated hydrochloric acid before extraction with ether. The low solubility of the trione in chloroform precluded the use of this solvent. The ultraviolet-absorption pattern of the tubes after countercurrent distribution (see p. 233) of root tissue extract from untreated plants was approximately the same whether chloroform or ether was used for the extraction. With shoot tissue, however, the ether extract showed intense maxima at 370 and 270 mμ in tubes 38–48, which were absent from a similar chloroform extract. The material responsible for this absorption was subsequently identified (Abbot, Grove & McCloskey, 1958) as kaempferol, 3:5:7:4′-tetrahydroxyflavone, which is virtually insoluble in chloroform.

The amine (II; R = NH_2). The macerated roots and shoots which had already been extracted with chloroform were separately stirred for 24 hr. with 2 N-hydrochloric acid (1 l.). Under these conditions any residual amine would probably have been converted into the trione (II; R = OH). After filtration the acid solution was extracted with ether (acidic fraction) and the aqueous layer was then made alkaline by the addition of sodium hydroxide (cooling) and re-extracted with ether (basic fraction). The acidic and basic fractions were recovered and their trione and amine contents estimated as described below. Neither fraction from either shoots or roots showed any specific absorption maxima near 290 mμ, indicating the absence of both amine and trione.

Griseofulvin. In view of the failure to detect griseofulvin metabolic products in the decay experiments (pp. 238 and 239) the shoot and root macerates which had previously been extracted with chloroform were separately agitated for 24 hr. with sodium hydrogen carbonate. After acidification, ether extraction and recovery the extracts were separated into acidic and neutral fractions and compared with controls from untreated plants. No differences were seen and there was no specific absorption between 250 and 350 mμ in any of the fractions.

Calculation of uptake

Crowdy *et al.* (1956) showed that two processes were involved in the uptake of griseofulvin by broad bean: an initial rapid entry into the roots which quickly attained the griseofulvin concentration of the external solution, followed by a prolonged uptake linearly related to the transpiration. For treating solutions of concentration 25 μg./ml. the mean of several experiments showed that transpiration uptake was 18 μg./ml. of water transpired, although deviations from this mean were considerable. For lower concentrations, the transpiration uptake (μg./ml. of water transpired) was taken as proportional to the concentration of the treating solution. In the present series of experiments with griseofulvin relatives uptakes have been calculated from the fresh weight of roots and weight of water transpired, assuming that the same considerations hold.

If x is the fresh weight of roots, y the weight of solute taken, v the volume of the treating solution, t the volume of water transpired, and C a constant, then

$$\text{Initial uptake} = y\left(\frac{x}{v+x}\right).$$

$$\text{Transpiration uptake} = \left[y\left(1-\frac{x}{v+x}\right)\right]\frac{Ct}{v} = \frac{yCt}{v+x}.$$

$$\text{Total uptake} = y\left(\frac{Ct+x}{v+x}\right).$$

For a 25 μg./ml. solution, $C = 0{\cdot}72$.

Uptakes calculated in this way are set out in Table 2; they are compared with the spectrophotometric estimations of uptake in Part 4 (Crowdy *et al.* 1959).

Spectrophotometric estimation and countercurrent distribution

With the exception of the trione (II; R = OH) all compounds obeyed Beer's law in the concentration range 10^{-3}–10^{-5} M, both in ethanol and in the upper phase of the carbon tetrachloride–methanol–water system used for countercurrent distribution. Absorption maxima and the corresponding molecular extinction coefficients for the eleven compounds are recorded in Table 3. The form of the

Table 2. *Uptake of griseofulvin relatives by bean plants*

The transpiration data are for the 3-day treatment of 100 bean plants. For the calculation of uptake see text.

Compound	Expt.	Treating solution: Vol. (ml.) v	Treating solution: Concn. (mg./l.) y/v	Final pH	Water transpired (ml.) t	Fresh wt. of roots (g.) x	Calculated uptake (mg.) $y\left(\frac{Ct+x}{v+x}\right)$
ld-(II; R = OMe)		5000	25	—	2453	185	51
(VI; R = OMe)		5000	25	—	1167	255	28
(IV)		5000	25	—	1355	221	31
(II; R = OPr^n)		5000	5	5·7	1858	202	10
(II; R = OBu^n)	*A*	4000	20	—	1395	303	25
	B	5000	5	—	1498	426	9
(V)		5000	25	—	2351	228	46
(III; R = OH)		4600	25	—	2078	162	43
(III; R = H)		5000	25	—	2183	385	45
(II; R = OH)	*A*	5000	20	—	1655	490	34
	B	5000	25	—	4785	371	96
(II; R = NH_2)		5000	25	6·3	1827	340	41
(I)	*A*	5000	15	—	1688	424	23
	B	16 000	25	4·85	6831	745	147

absorption curve for the acidic trione (II; R = OH) in ethanol, and in particular the intensity of the band near 290 mμ, varies with concentration due to dissociation of the compound in the ionizing solvent. For this reason the spectrophotometric estimation of the trione was carried out in 0·1 N-sodium hydroxide in which dissociation was essentially complete and the anion might be assumed to be the only molecular species present. In this medium it was found that Beer's law held within about 3% for solutions in the range 10^{-4}–10^{-5} M.

The carbon tetrachloride–methanol–water (62:35:3, by vol.) system (Crowdy *et al.* 1955) was used for countercurrent distribution of all the compounds studied. The mobile phase was the methanol-rich upper phase. In this system the partition ratios of most of the compounds were such as to bring them into a range of tubes uncontaminated by the carbon tetrachloride-soluble green pigments or methanol-soluble brown pigments. The more polar compounds, however, notably the trione (II; R = OH), the amine (II; R = NH_2) and the diol (III; R = OH), appeared in tubes heavily contaminated by phenolic and other brown pigments; this made estimation and recovery difficult, but the advantages of retaining the same solvent system throughout outweighed the disadvantages encountered with these three compounds.

Data for 50 transfers with the pure compounds are included in Table 3. For the purposes of this paper K is defined as the partition ratio actually operating during the distribution, and was obtained from the equation

$$N = nK/(K+1),$$

where n is the total number of transfers and N the number of the equilibrium tube containing the maximum amount of the compound under investigation. N was determined experimentally in the usual way.

For all the compounds studied the experimentally determined distribution curve was found to be in good agreement with the theoretical distribution calculated (Williamson & Craig, 1947) from the value of K determined in this way. The fraction (T_N) of the total solute appearing in tube N and the location of the batch of tubes containing 0·99 of the total solute are also given in Table 3. It was assumed that the constituents of the extracts of macerated root and shoot tissue would have a negligible effect on the distribution; this was shown to be true for griseofulvin (cf. Crowdy *et al.* 1955).

Estimation of griseofulvin relatives in treating solutions

Concentration of griseofulvin solutions *in vacuo* at 60° before extraction with chloroform was shown (Crowdy *et al.* 1955) to cause appreciable losses of griseofulvin, for which correction was made. In the present series of experiments the bulked root washings and treating solution were extracted without prior concentration. The residual aqueous solution was then examined spectrophotometrically (l, 4 cm.) to ensure that extraction was complete. Under these conditions griseofulvin (90 mg.) was recovered quantitatively from water (5 l.) by extraction with chloroform (3 × 2 l.). Chloroform was effective in extracting quantitatively all the neutral compounds with the exception of the diol (III; R = OH), where a final extraction with ether was necessary. In certain cases, notably the dechloro analogue (VI; R = OMe), 7-chloro-4:6:4′-trimethoxy-6′-methylgris-3′-en-3:2′-dione (IV) and the propyl and butyl homologues of griseofulvin, where the presence of metabolites was known or suspected, the recovered solute was dissolved in ether and separated into neutral, acidic and phenolic fractions by extraction with aqueous sodium hydrogen carbonate followed by 2 N-sodium hydroxide and recovery. The neutral fraction was dissolved in benzene and chromatographed in ultraviolet light on alumina. After elution of coloured impurities with benzene, the blue fluorescent band was eluted with benzene–methanol and the compound under investigation estimated spectrophotometrically in the eluate after recovery and dissolution in ethanol. It was then recovered and identified chemically. The acidic and phenolic fractions were investigated

separately (see below). With the remaining neutral compounds, namely the griseofulvin *ld*-diastereoisomer, 7-chloro-6:2′-diethoxy-4-methoxy-6′-methylgris-2′-en-3:4′-dione (V), the alcohols (III; R = OH) and (III; R = H) and grisan-3:4′-dione, the chloroform extract was chromatographed without separation of acidic and phenolic fractions and the compound estimated spectrophotometrically. With the exception of grisan-3:4′-dione, all compounds were recovered and identified, and the weight recovered agreed reasonably well with that estimated spectrophotometrically. Special methods of extraction and estimation were necessary for the trione (II; R = OH) and the amine (II; R = NH_2).

The trione (II; R = OH). The treating solution and root washings were acidified (Congo red) with concentrated hydrochloric acid and continuously extracted with ether. The solute from the bulked ether extracts was dissolved in 0·1 N-sodium hydroxide and the trione content estimated spectrophotometrically. After acidification and re-extraction with ether the trione was recovered and characterized chemically. The intensity of the absorption band at 290 mμ was such that interference from impurities exuded by the roots was negligible by comparison with the trione absorption as judged by the ratio of the band intensities at 290 and 330 mμ. Thus purification on a Celite column was unnecessary.

The amine (II; R = NH_2). In aqueous media the amine is hydrolysed to the trione (II; R = OH) and hence the treating solution was analysed for both components. It was impracticable to separate relatively large quantities of the amine from the other constituents by extraction with dilute hydrochloric acid, as decomposition to the trione (II; R = OH) is rapid under these conditions. The favoured procedure was to remove impurities from the amine; the treating solution was extracted with chloroform and the latter was extracted with aqueous sodium hydrogen carbonate. The acid fraction and the residual solute in the chloroform extract were recovered and the trione and amine contents estimated in 0·1 N-sodium hydroxide and in ethanol respectively.

Detection and estimation of metabolites in the treating solutions

Weights estimated spectrophotometrically are preceded by ≡, e.g. (≡ 1·5 mg.).

Compound (VI; R = OMe). The acidic fraction showed a sharp absorption maximum at 288 mμ (in 0·1 N-sodium hydroxide). Chromatography of the solute in ether on Celite as described on p. 231 gave a gummy product (1·5 mg.) which contained the material with λ_{max} 288 mμ (≡ 1·5 mg.) but which could not be crystallized. It was assumed to be 4:6-dimethoxy-2′-methylgrisan-3:4′:6′-trione (VI; R = OH) ($\lambda_{max.}$ 289 mμ, ε 45 750 in sodium hydroxide), produced by hydrolysis of (VI; R = OMe).

The phenolic fraction contained a trace of an unidentified metabolite with $\lambda_{max.}$ 285 mμ.

Compound (IV). The acidic fraction showed maxima at 272, 290 and ~ 325 mμ (~ represents an inflexion) in ethanol, but the metabolite (3 mg.) could not be identified and the fraction was not examined further.

The phenolic fraction showed a weak maximum at 288 mμ, due possibly to a trace of a second metabolite.

Compound (I). Experiment (*B*) is described here and in subsequent sections.

Table 3. *Molecular extinction coefficients and countercurrent-distribution data for griseofulvin relatives*

Extinctions were measured in ethanol and in the upper phase of the countercurrent distribution system [carbon tetrachloride–methanol–water (62:35:3, by vol.)]. Fifty transfers were made in the distribution. For definition of symbols see text.

	Molecular extinction coefficients determined in									Countercurrent-distribution data			
	Ethanol				Upper phase								
Compound	$\lambda_{1\,max.}$ (mμ)	ϵ_1	$\lambda_{2\,max.}$ (mμ)	ϵ_2	$\frac{\epsilon_1}{\epsilon_1-\epsilon_2}$	$\lambda_{1\,max.}$ (mμ)	ϵ_1	$\lambda_{2\,max.}$ (mμ)	ϵ_2	$\frac{\epsilon_1}{\epsilon_1-\epsilon_2}$	K	N	T_N	Tube numbers (range) containing 99% of total solute
ld-(II; R = OMe)	291	23 500	325	5850	1·33	293	24 250	327	6175	1·34	1·56	30·5	0·114	22–39
(VI; R = OMe)	287	25 500	~ 320	5570	1·28	288	25 750	~ 325	5610	1·28	1·38	29	0·114	21–38
(IV)	291	19 750	326	5100	1·35	291	20 750	326	5220	1·34	1·70	31·5	0·114	24–40
(II; R = OPr^n)	292	22 000	325	4975	1·30	294	23 250	326	5750	1·33	1·33	28·5	0·114	20–37
(II; R = OBu^n)	292	23 500	325	5200	1·29	294	24 000	328	5650	1·31	1·17	27	0·113	19–36
(V)	293	23 000	327	5100	1·29	295	23 500	328	5400	1·30	1·22	27·5	0·112	19–36
(III; R = OH)	288	20 750	323	5130	1·33	289	23 750	324	5420	1·30	5·41	42·2	0·151	35–49
(III; R = H)	289	19 500	324	4470	1·30	290	20 500	325	4750	1·30	2·00	33·3	0·118	25–41
(II; R = OH)	290*	44 000*	330*	6400*	1·17*	294	21 500‡	328	5280‡	1·32	3·20	38	0·131	31–45
(II; R = NH_2)	290	47 250	330	6750	1·17	290	46 000	~ 330	6500	1·16	5·25	42	0·153	36–48
(I)	250	10 470	328	5129	1·96	—	†	328	4940	—	1·10	26·2	0·112	18–35

* In 0·1 N-sodium hydroxide. † Inaccurate in this solvent. ‡ 1·6 μM-solution.

The neutral chloroform extract, a brown granular solid (360 mg.), was extracted with hot benzene and the soluble material, a brown gum (260 mg.), separated by chromatography on alumina in benzene–methanol (199:1 rising to 99:1, v/v) into two fractions:

(i) A gum (140 mg.) containing a component, $\lambda_{max.}$ 325 mμ, possibly grisan-3:4′-dione ($\equiv$ 5 mg.). A positive identification was not obtained, however, despite further treatment, and only an intractable precipitate was obtained with Brady's reagent.

(ii) A gum (100 mg.) containing a component, $\lambda_{max.}$ 282, 289 mμ. After sublimation at 150°/10^{-1} mm. the product was combined with a similar fraction obtained by sublimation, at 150°/10^{-1} mm., of the benzene-insoluble material, and the total sublimate (83 mg.) crystallized from benzene, yielding colourless needles (67 mg.), m.p. 168–188°; $[\alpha]_D^{22}$ +68·5° in ethanol (*c*, 1·1) (Found: C, 71·3; H, 7·3. $C_{13}H_{16}O_3$ requires C, 70·9; H, 7·3%), light-absorption in ethanol, $\lambda_{max.}$ 282, 290 mμ, ϵ 3240, 2870 respectively, of a mixture of α- and β-grisan-3:4′-diols in almost equal proportion, identified by comparison of the infrared spectrum (Nujol mull) with that of a synthetic mixture prepared from (±)-α- and (±)-β-grisan-3:4′-diols (McCloskey, 1958). The specific rotation of the natural mixture indicates that only the (+)-diols are present, since (+)-α-grisan-3:4′-diol has $[\alpha]_D^{21}$ +73° in ethanol (*c*, 1·0).

The unsublimed residue (33 mg.) of the benzene-insoluble material (above) was extracted twice with hot ethanol and once with boiling benzene, and the final insoluble residue (5 mg.), m.p. >300°, was rejected. Evaporation of the combined extracts yielded a gum which was separated by treatment with warm ethanol into a soluble gum (8 mg.; $\equiv$ 6 mg. of grisan-3:4′-diol), and an insoluble solid (20 mg.), m.p. 186–194° [Found: C, 75·8; H, 7·2%; *M* (Rast) 1970], light-absorption in ethanol, $\lambda_{max.}$ 282, ~289 mμ, $E_{1\,cm.}^{1\%}$ 110, 95 respectively. The infrared spectrum showed bands attributable to an aromatic ring, but no hydroxyl or carbonyl bands.

The amount of grisan-3:4′-diol present in the residual treating solution was estimated spectrophotometrically as 93 mg.

Estimation of griseofulvin relatives in plant-tissue extracts

In the early work on the determination of griseofulvin in shoot-tissue extracts (Crowdy *et al.* 1955) it was possible to estimate the griseofulvin content directly from the values of the extinctions in the equilibrium tubes after countercurrent distribution. In contrast with these observations, more recent work on extracts of untreated plants has shown that the background absorption at 291 and 326 mμ in tube 33 does not always have the same value, and that with certain extracts the background absorption begins to rise steeply with increasing tube number well in advance of tube 33. This was first noticed in decay experiments (Crowdy *et al.* 1956), but was subsequently found to be of fairly general occurrence. The reason for these variations in background absorption is not known, although seasonal influences may be involved. Acidic and particularly phenolic components are mainly responsible for the background absorption in untreated plant extracts.

Where the simple procedure outlined by Crowdy *et al.* (1955) was unsatisfactory, it was generally found that the background absorption in tube 33 was linear over the wavelength range 270–320 mμ; the extinction due to griseofulvin was then calculated by using the three-point correction procedure of Morton & Stubbs (1946).

Alternatively, the solute in tubes 25–41 was recovered and separated into neutral, phenolic and acidic components and the griseofulvin content of the neutral fraction estimated spectrophotometrically after chromatography on alumina. This last general method of analysis of shoot-tissue extracts was adopted for all the neutral compounds in the present study and was satisfactory. An identical procedure was adopted for root-tissue extracts.

Extinctions E_1 and E_2 were measured at two wavelengths, λ_1 and λ_2 (Table 3) and the ratio E_1/E_2 compared with ϵ_1/ϵ_2 for the pure compound. Agreement was usually good; but where appreciable background absorption was suspected the observed extinctions were corrected by the procedures of Crowdy *et al.* (1955) (constant background) or Morton & Stubbs (1946) (linear variation with wavelength) as appropriate. In the former method the corrected extinction at λ_1 is given by $\epsilon_1(E_1 - E_2)/(\epsilon_1 - \epsilon_2)$. Values of $\epsilon_1/(\epsilon_1 - \epsilon_2)$ are given in Table 3.

Special methods of estimation were again necessary for the trione (II; R = OH) and the amine (II; R = NH_2).

The trione (II; R = OH). The solute from tubes 31–45 was dissolved in ether and was separated into acidic, phenolic and neutral fractions in the usual way. The trione content of the acidic fraction was estimated spectrophotometrically in 0·1 N-sodium hydroxide either directly or after chromatography on Celite. Thus in a trial estimation the trione (10·0 mg.) added to shoot tissue extract (2·84 g.) from untreated plants was subjected to 50 transfers. The solute from tubes 31–45 was dissolved in ether (200 ml.) and extracted with aq. sodium hydrogen carbonate (2 × 50 ml.). The acid fraction on recovery was redissolved in ether (200 ml.) and chromatographed on Celite (5 g.), buffered at pH 7·0, by the fractional elution technique (eluent: ether, 200 ml. portions). Eluates III–VI contained the trione together with some impurities: spectrophotometric estimation of the trione in 0·1 N-sodium hydroxide gave a high value ($\equiv$ 12·0 mg.).

Similar, but slightly more accurate, results were obtained with root-tissue extract in place of shoot-tissue extract.

The amine (II; R = NH_2). The solute from tubes 36–48 was dissolved in ether and the basic fraction separated by extraction at 0° with 2 N-hydrochloric acid. This procedure, by which the amine is separated from other constituents, is the reverse of that found most suitable for analysis of the amine in the treating solution, but was adopted as the quantities involved were small. It led to some decomposition of the amine, and the acid extract (made alkaline) after recovery of the basic fraction by ether extraction contained some of the trione (II; R = OH). This was estimated separately in the usual way. The amine content of the basic fraction was estimated spectrophotometrically in ethanol. In a trial estimation, the amine (5·0 mg.) added to shoot-tissue extract (1·0 g.) from untreated plants was subjected to 50 transfers and tubes 36–48 were analysed as described above with the following results: (II; R = NH_2) (basic fraction), 1·5 mg.; (II; R = OH) (acid fraction), 2·0 mg., (by decomposition during isolation of basic fraction), 0·8 mg.; total 4·3 mg.

There is no decomposition of the amine to the trione during the actual countercurrent distribution: the trione

appearing in the acid fraction must therefore arise by decomposition of the amine during the extraction with 2N-hydrochloric acid. The amount of decomposition was not strictly reproducible.

A similar almost quantitative estimation of the amine (5·0 mg.) in root-tissue extract (500 mg.) was also achieved.

In the following sections weights of griseofulvin relatives or metabolites estimated spectrophotometrically in individual fractions after countercurrent distribution are preceded by (≡). To obtain the total weight present in the tissue extract a correction has sometimes to be applied to allow for the weight of the compound, calculated to be present from the distribution data (Table 3), in adjacent batches of tubes. This allowance has been made in the balance sheets presented in the next paper (Part 4).

Extraction and identification of compounds from shoot tissue and search for metabolites

In order to establish the validity of the spectrophotometric methods of estimation it was necessary to recover the compounds in question and identify them chemically (Crowdy *et al.* 1955). In the majority of cases this had been done and the experimental procedures are described below. The weight of each compound recovered has been recorded and may be compared with the weight believed to be present from spectrophotometric estimation. The recovery of some compounds proved to be more difficult than the recovery of griseofulvin (Crowdy *et al.* 1955) and only a fraction of the weight believed to be present from the spectrophotometric estimation was actually recovered. In no case, however, was a second component with similar absorption maxima in the ultraviolet isolated from the same fraction.

At the same time a search was made for metabolites with the aromatic ring intact by scanning the tubes after countercurrent distribution and all fractions derived therefrom for specific ultraviolet absorption above 250 mμ, and by comparing the spectra obtained with the spectra of similar fractions derived from untreated plants. Major deviations from the absorption pattern found in the control experiment are summarized in Table 4. Details are also given of the search for metabolites after the griseofulvin-decay experiments (Crowdy *et al.* 1956). Except where stated, all fractions were examined in ethanol solution. With the exception of metabolites of grisan-3:4′-dione the amount of unidentified major metabolites present has been estimated roughly, assuming a molecular weight of 350 and $\epsilon = 25\,000$.

Compound ld-(II; R = OMe). Tubes 0–25 and 37–50 from the countercurrent distribution were similar to the control and were not investigated. Tubes 26–36 showed specific absorption at 285 mμ and the solute was separated into acidic, phenolic and neutral fractions in the usual way.

The phenolic fraction was similar to the control but the acidic fraction had absorption maxima at 282 and 325 mμ. The amount present was very small. The neutral fraction, showing $\lambda_{max.}$ 285 mμ, was dissolved in benzene and chromatographed on alumina in the usual way. Benzene:methanol (200:1, v/v) eluted material with $\lambda_{max.}$ 288 mμ (≡ 2 mg.) and this was sublimed at 190°/0·01 mm. Crystallization of the sublimate from ethanol afforded

Table 4. *Recovery of griseofulvin relatives from bean plants and detection of metabolites*

Griseofulvin relatives and metabolites were detected by differences in the ultraviolet-absorption spectra of extracts of treated and untreated plants. Tube numbers refer to a 50-transfer countercurrent distribution (for details see text).

	Shoots		Roots	
Compound	Recovery	Metabolites	Recovery	Metabolites
ld-(II; R = OMe)	++	Acidic, tubes 26–36, $\lambda_{max.}$ 282, 325 mμ	++	—
(VI; R = OMe)	++	Acidic, tubes 24–33, $\lambda_{max.}$ 280, 325 mμ	++	—
(IV)	+	—	–	3-Chloro-2-hydroxy-4:6-dimethoxybenzoic acid Neutral, tubes 24–35, $\lambda_{max.}$ 291 mμ Acidic, tubes 24–35, m.p. 284–286°, $\lambda_{max.}$ 287, ~330 mμ Neutral, tubes 36–50, $\lambda_{max.}$ 262, 290 mμ Acidic, tubes 36–50, $\lambda_{max.}$ ~285 mμ
(II; R = OPr^n)	+	—	++	—
(II; R = OBu^n)	+	—	++	Acidic, tubes 34–50, $\lambda_{max.}$ 287 mμ
(V)	++	Acidic, tubes 20–33, $\lambda_{max.}$ 282, 325 mμ	++	—
(III; R = OH)	+	Acidic, tubes 35–50, $\lambda_{max.}$ 282, 320 mμ	++	—
(III; R = H)	++	—	++	—
(II; R = OH)	–	—	–	—
(II; R = NH_2)	+	Acidic, tubes 33–45, $\lambda_{max.}$ 282, 320 mμ	+	Neutral, tubes 28–36, $\lambda_{max.}$ 285 mμ Acidic, tubes 28–47, $\lambda_{max.}$ 290 mμ
(I)	–	Acidic, tubes 20–32, $\lambda_{max.}$ 280, 322 mμ	–	Neutral, tubes 21–31, $\lambda_{max.}$ 281, ~288 mμ
		α- and β-4′-Hydroxygrisan-3-ones (+)-α-Grisan-3:4′-diol Neutral, tubes 33–50, $\lambda_{max.}$ 325 mμ		α- and β-Grisan-3:4′-diols

++ = Present; positive identification. + = Presence inferred from strong evidence. – = Absent.

(1) needles, m.p. 236–40° raised to 256° by repeated crystallization from ethanol, and (2) an amorphous powder, m.p. 129–190°. The compound (m.p. 256°) is a normal constituent of the broad-bean shoot and more especially of the root; it was obtained in this fraction in all experiments and was identified as betulin (Abbot *et al.* 1958).

The amorphous powder (2) was separated into ketonic and non-ketonic fractions by treatment with Girard's reagent P. The ketonic fraction ($\lambda_{max.}$ 292 mμ) crystallized from ethanol in needles (1 mg.), m.p. and mixed m.p. with *ld*-7-chloro-4:6:2′-trimethoxy-6′-methylgris-2′-en-3:4′-dione, 218–220°.

In subsequent descriptions in this and the following sections, only data for tubes and fractions which differed significantly from the control will be described. Fractions not so mentioned were similar to the control.

Compound (VI; R = OMe). Tubes 24–33: the acid fraction contained a trace of an acidic substance with $\lambda_{max.}$ 325 and 280 mμ.

The neutral fraction (≡ 8 mg.) was extracted with boiling benzene and the solution chromatographed, elution being effected with benzene:methanol (99:1, v/v). Eluates showing absorption maxima at 287 mμ were combined and the solute, after washing with ether, crystallized from methanol in needles (4·5 mg.) of 4:6:2′-trimethoxy-6′-methylgris-2′-en-3:4′-dione, m.p. 180–181°, undepressed on admixture with authentic material.

Compound (IV). Tubes 25–37: the neutral fraction showed a maximum at 288 mμ with an inflexion at 265 mμ. After alumina chromatography a gummy eluate was obtained showing maxima at 261 and 290 mμ (≡ 1 mg.), characteristic of 7-chloro-4:6:4′-trimethoxy-6′-methylgris-3′-en-3:2′-dione (Grove *et al.* 1952*a*), but a positive identification could not be obtained.

Compound (II; R = OPr^n). Tubes 21–35: chromatography of the neutral fraction yielded a very impure eluate with $\lambda_{max.}$ 291 mμ (≡ 0·3 mg.). The solute was recovered and separated into ketonic and non-ketonic portions by treatment with Girard's reagent P. The ketonic fraction did not crystallize from ether and the 291 mμ-absorbing component was not formally identified.

Compound (II; R = OBu^n). Expt. *A* is described here and in the following section.

Tubes 22–30: the neutral fraction showed weak specific absorption at 290 mμ and was chromatographed on alumina. Elution of a blue fluorescent band with benzene–methanol (99:1, v/v) afforded some concentration of this material, but only a trace (<0·5 mg.) was present and precluded a formal identification.

Compound (V). Tubes 20–33: the neutral fraction, $\lambda_{max.}$ 290 mμ (≡ 3 mg.), was chromatographed on alumina and the eluates containing the 290 mμ-absorbing material were combined and the solute sublimed at 115°/10^{-2} mm. The sublimate was treated with ether and the more soluble portion crystallized from ether–light petroleum (b.p. 40–60°) in small prisms (0·5 mg.), identified as 7-chloro-6:2′-diethoxy-4-methoxy-6′-methylgris-2′-en-3:4′-dione by mixed m.p. (165–170°) and comparison of infrared spectra.

The acidic fraction contained a trace of a substance with $\lambda_{max.}$ 282 and 325 mμ.

Compound (III; R = OH). Tubes 35–50: the acidic fraction had specific absorption maxima at 282 and 320 mμ.

The neutral fraction showed a maximum at 288 mμ. The ether soluble portion was chromatographed on alumina in ether and eluates exhibiting maxima at 288 mμ (≡ 0·1 mg.) were sublimed giving an intractable solid, m.p. 130–160°.

Compound (III; R = H). Tubes 27–37: the neutral fraction showed $\lambda_{max.}$ 287 mμ (≡ 5 mg.), and after separation of insoluble amorphous material was chromatographed on alumina in ultraviolet light. Elution of the blue fluorescent band with benzene–methanol (99:1, v/v) gave a yellow gum which was sublimed at 170°/0·1 mm. The sticky yellow sublimate was treated with methanol, insoluble material rejected, and the solute, after recovery, crystallized from benzene:light petroleum (b.p. 60–80°). Three crystallizations gave 7-chloro-6′-hydroxy-4:6-dimethoxy-2′-methylgrisan-3-one (1 mg.), m.p. and mixed m.p. 200–203°.

Compound (II; R = OH). In Expt. *A* in which the tissues were macerated in water a careful investigation of all tubes after countercurrent distribution, and of all fractions derived therefrom, failed to reveal the presence either of the trione or of any metabolites. Accordingly, in experiment *B* a very high uptake was arranged (Table 2), and the tissues were macerated in aq. sodium hydrogen carbonate (p. 232). All tubes and fractions were carefully examined, but there was no trace either of the trione or of metabolites.

Compound (II; R = NH_2). Tubes 33–45: the acidic fraction showed strong peaks at 282 and 320 mμ (293 and 342 mμ in 0·1 N-sodium hydroxide) (≡ 9 mg.). The material responsible for this absorption was not identified.

Tubes 46–50: the acidic fraction had a weak peak at 290 mμ in 0·1 N sodium hydroxide. The compound responsible for this absorption could not be obtained in crystalline form: if it was the trione (II; R = OH) less than 1 mg. was present. The trione is not found in this batch of tubes, and if present must have been produced by decomposition of the amine during recovery of the solute or separation of the fractions. On this admittedly slender evidence a trace of 2′-amino-7-chloro-4:6-dimethoxy-6′-methylgris-2′-en-3:4′-dione may have been present in the shoots.

Compound (I). Tubes 20–32: the acidic fraction (6 mg.) showed peaks at 280 and 322 mμ, but purification was not attempted.

The neutral fraction (500 mg.), $\lambda_{max.}$ 325 mμ, was extracted with ether and the recovered extract chromatographed on alumina in benzene. Elution with benzene–methanol afforded a gum (59 mg.), containing a component $\lambda_{max.}$ 327 mμ (≡ apparent 7 mg. of grisan-3:4′-dione) which passed into the non-ketonic fraction on treatment with Girard's reagent P, but further purification by fractional sublimation up to 150°/0·1 mm. was ineffective and the gums could not be crystallized. However, the infrared spectrum (Nujol mull) of the main fraction was fairly similar, in the carbonyl and hydroxyl regions, to the spectra of both α- and β-4′-hydroxygrisan-3-one (McCloskey, 1958), and it seems likely that both these products are present as metabolites.

Tubes 33–50: the neutral fraction, a partly crystalline gum (141 mg.), showed a strong peak at 282, ~290 mμ (≡ 33 mg. grisan-3:4′-diol) and was chromatographed on alumina in benzene. Fractional elution with benzene–methanol (199:1, v/v) gave a component, $\lambda_{max.}$ 325 mμ (≡ 6 mg., apparent grisan-3:4′-dione), which was not examined, and a component, $\lambda_{max.}$ 282, ~290 mμ (≡ 34 mg. grisan-3:4′-diol), a portion of which (30 mg. ≡ 16 mg. of

diol) was sublimed at 165°/10^{-1} mm. The sublimate was washed with ether and then crystallized from benzene, yielding needles (12 mg.) of α-grisan-3:4′-diol, m.p. 187–198°, $[\alpha]_D^{22}$ +79° in ethanol (*c*, 0·6; microdetermination on 1·5 mg.) (Found: C, 70·4; H, 7·3. $C_{12}H_{16}O_3$ requires C, 70·9; H, 7·3%). The infrared spectrum (Nujol mull) was identical with that of authentic (+)-α-grisan-3:4′-diol, $[\alpha]_D^{21}$ +73° in ethanol (*c*, 1·0) (McCloskey, 1958). Countercurrent distribution (n = 50) of (±)-α-grisan-3:4′-diol gave K = 3·35 and N = 38·5; tubes 30–46 contained 0·99 of the total diol distributed.

Compound dd-(II; R = OMe). The 14-day decay experiment (Crowdy *et al.* 1956) is described here but the 6·5-day decay experiment was almost identical. There was little difference between the controls corresponding to the 3-day uptake and the decay experiments.

Tubes 26–36: the acidic fraction contained a small amount of a substance with $\lambda_{max.}$ 290 and 320 mμ.

The neutral fraction had $\lambda_{max.}$ 285 mμ (≡ 4 mg.) and was chromatographed on alumina. Elution of the blue-fluorescent band with benzene–methanol (99:1, v/v) gave, after recovery, sublimation *in vacuo*, treatment with light petroleum and crystallization from ethanol, griseofulvin (2 mg.) prisms, m.p. 212–215°, undepressed by admixture with an authentic specimen.

Tubes 37–50: the acidic fraction showed $\lambda_{max.}$ 285 and 320 mμ (≡ 13 mg.) ($\lambda_{max.}$ 293 and 342 mμ in 0·1 N-sodium hydroxide). Considerable purification was effected (accompanied by appreciable losses of material) by molecular distillation, chromatography on Celite and fractional precipitation from solvents, but the substance absorbing at 285 and 320 mμ was not obtained in crystalline form. A quantitative determination of halogen in the final purified fraction (5 mg.) gave Cl, 0·84%. A corresponding determination on the control fraction gave Cl, 1·2%.

Extraction and identification of compounds from root tissue and search for metabolites

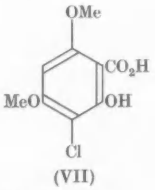
Compound ld-(II; R = OMe). Tubes 24–36: the neutral fraction showed $\lambda_{max.}$ 290 mμ (≡ 13 mg.) and was separated into non-ketonic and ketonic fractions in the usual way. The ketonic material crystallized from ethanol in needles (5 mg.), m.p. and mixed m.p. with *ld*-7-chloro-4:6:2′-trimethoxy-6′-methylgris-2′-en-3:4′-dione, 210–215°.

Compound (VI; R = OMe). Tubes 23–35: the neutral fraction had a sharp peak at 286 mμ (≡ 8 mg.). Repeated sublimation of the material at 120–150°/0·1 mm. and treatment of the sublimate with ether, followed by crystallization of the more soluble component from ether–methanol, afforded 4:6:2′-trimethoxy-6′-methylgris-2′-en-3:4′-dione (0·5 mg.; m.p. and mixed m.p. 177–180°).

Compound (IV). The tubes, after countercurrent distribution, showed a well-defined peak at 290 mμ with a shoulder at 265 mμ, first obvious in tube 24 and rising to maximum intensity in tube 31. Thereafter the intensity of the peak declined, eventually merging with the shoulder to form a single broad maximum at 275 mμ which persisted almost to tube 50.

Tubes 24–35: the acidic fraction showed $\lambda_{max.}$ 286, ~332 mμ (≡ 9 mg.) ($\lambda_{max.}$ 294, ~335 mμ in 0·1 N-sodium hydroxide). Treatment of the crude material (17 mg.) with benzene–ether yielded a phenolic acid (3 mg.), m.p. 284–288° (decomp.) (gas evolution), ($\lambda_{max.}$ 287, ~330 mμ, $E^{1\%}_{1\,cm.}$ 625, 122 respectively), soluble, with evolution of CO_2 in sodium hydrogen carbonate. There was insufficient material for further purification. The infrared spectrum showed C=O bands at ~1709 and 1685 cm.$^{-1}$ in chloroform solution, and this together with the ultraviolet spectrum suggested the presence of a 4-hydroxycoumaranone chromophore (Duncanson *et al.* 1957).

Sublimation at 215°/0·0001 mm. of the gummy residue obtained by evaporation of the mother liquors gave a sublimate which, on treatment with ether, afforded a solid (1 mg.), m.p. and mixed m.p. with 3-chloro-2-hydroxy-4:6-dimethoxybenzoic acid (VII), 213–218° (decomp.). Under the standard conditions used for countercurrent distribution (n = 50), 3-chloro-2-hydroxy-4:6-dimethoxybenzoic acid has K = 2·03 and N = 33·5. The ultraviolet-absorption curve shows maxima at 221, 265, and 306 mμ and in the crude acidic fraction these peaks were presumably masked by the much greater amount of the component, m.p. 284–288° (decomp.), $\lambda_{max.}$ 287 mμ.



(VII)

The phenolic fraction showed $\lambda_{max.}$ 288 mμ (≡ 6 mg.) and was sublimed at 200°/0·0001 mm. The sublimate crystallized from acetone in needles (1 mg.) m.p. 218–224° (decomp.) of 3-chloro-2-hydroxy-4:6-dimethoxybenzoic acid (see above). From the ultraviolet absorption this was a minor component only of the phenolic fraction: the major component ($\lambda_{max.}$ 288, ~330 mμ) could not be identified, but it was possibly the same as the major component, m.p. 284–288° (decomp.) of the acidic fraction.

The neutral fraction showed a single maximum at 289 mμ (≡ 7 mg.). Sublimation at 200°/0·1 mm. and chromatography of the sublimate afforded a gum (12 mg.) which had a sharp absorption band at 291 mμ but could not be solidified. The infrared spectrum showed a broad OH band and a broad C=O band at 1725 cm.$^{-1}$.

Tubes 36–50: the acidic fraction had a weak maximum at 285 mμ.

The neutral fraction contained a trace of material absorbing at 290 and 262 mμ, but the quantity was too small for further investigation.

Compound (II; R = OPr^n). Tubes 21–35: the neutral fraction had λ_{max} 290 mμ (≡ 3 mg.). Chromatography on alumina and elution of the blue fluorescent band with benzene:methanol (200:1, v/v) yielded a solid which was separated into ketonic and non-ketonic fractions. The ketonic fraction crystallized from ether in micro-needles (<0·2 mg.), identified as 7-chloro-4:6-dimethoxy-6′-methyl-2′-*n*-propoxygris-2′-en-3:4′-dione by comparison of the infrared spectrum with that of an authentic specimen.

Compound (II; R = OBu^n). Tubes 20–33: the neutral fraction showed $\lambda_{max.}$ 292 mμ (≡ 6 mg.). Chromatography followed by fractional crystallization of the solute from light petroleum (b.p. 60–80°) afforded 2′-*n*-butoxy-7-chloro-4:6-dimethoxy-6′-methylgris-2′-en-3:4′-dione (3 mg.), identified by comparison of the infrared spectrum with an authentic specimen.

Tubes 34–50: the acidic fraction had $\lambda_{max.}$ 287 mμ [≡ 1·5 mg. of the trione (II; R = OH)]. The gummy fraction was extracted with benzene, but the benzene-insoluble portion was intractable and a positive identification was not obtained. Compound (II; R = OH), if present, would have been produced before countercurrent distribution, and possibly during maceration.

Compound (V). Tubes 20–35: the neutral fraction showed $\lambda_{max.}$ 290 mμ (≡ 26 mg.) and was separated into ketonic and non-ketonic portions. The ketonic material crystallized from a small volume of methanol in needles (16 mg.), m.p. and mixed m.p. with 7-chloro-6:2′-diethoxy-4-methoxy-6′-methylgris-2′-en-3:4′-dione, 163–170°.

Compound (III; R = OH). After countercurrent distribution an absorption peak ($\lambda_{max.}$ 290 mμ) was observed in tubes close to tube 42.

Tubes 36–50: the neutral fraction had $\lambda_{max.}$ 288 mμ (≡ 4 mg.) and was chromatographed on alumina in ether. Eluates containing 288 mμ-absorbing material were bulked and the solute repeatedly sublimed at 150–180°/0·0001 mm. The final sublimate, a colourless gum, could not be obtained in crystalline form, but the infrared spectrum showed bands due to –OH and C=O and was generally similar to that of 7-chloro-4′;6′-dihydroxy-4:6-dimethoxy-2′-methylgrisan-3-one.

Compound (III; R = H). Tubes 27–37: the neutral fraction had $\lambda_{max.}$ 287 mμ (≡ 8 mg.) and was chromatographed in benzene on alumina. Elution with benzene:methanol (99:1, v/v) of the band fluorescing blue in ultraviolet light afforded a colourless solid (36 mg.) which was fractionally crystallized from ethanol. The more soluble fraction (18 mg.) was sublimed at 170°/0·1 mm. and the sublimate (11 mg.) repeatedly crystallized from benzene–light petroleum (b.p. 60–80°). Identification of the purified product (4 mg.), m.p. 203–205°, undepressed by admixture with 7-chloro-6′-hydroxy-4:6′-dimethoxy-2′-methylgrisan-3-one, was confirmed by comparison of the infrared spectra.

Compound (II; R = OH). As with the shoot tissue extracts (see p. 237), examination of the extracts from both experiments *A* and *B* failed to disclose the presence either of the trione or of metabolites.

Compound (II; R = NH_2). Tubes 28–36: the acidic fraction had $\lambda_{max.}$ 290 mμ [≡ 0·5 mg. of the trione (II; R = OH): see below].

The neutral fraction had a weak peak at 285 mμ. This metabolite was not identified but not more than 0·3 mg. could have been present. It was not eluted by benzene–methanol (98:2, v/v) from an alumina column and was not therefore 2′-acetamido-7-chloro-4:6-dimethoxy-6′-methylgris-2′-en-3:4′-dione ($\lambda_{max.}$ 289 mμ, ε 29 400) which is quantitatively eluted from such a column by benzene–methanol (99:1, v/v). The acetamido-compound was thought to be a possible metabolite in view of the work of Jones & Wignall (1955).

Tubes 37–47: the basic fraction showed specific absorption at 290 mμ (≡ 2 mg.). Attempts to crystallize and identify this substance as the amine (II; R = NH_2) were unsuccessful, but it is unlikely that a basic metabolite of the amine would be present.

The acidic fraction showed specific absorption at 290 mμ in 0·1N-sodium hydroxide [≡ 0·9 mg. of the trione (II, R = OH)]. The equivalent amount of trione, calculated from the theoretical distribution, was found in tubes 28–36 (which contain no amine), and the latter trione must have been present as such before countercurrent distribution. It seems probable therefore that both trione fractions were produced during maceration and extraction of the macerate.

Compound (I). Tubes 21–31: the neutral fraction (40 mg.) showed $\lambda_{max.}$ 281, ~288 mμ (≡ 10 mg. of apparent grisan-3:4′-diol) and was chromatographed on alumina in benzene. Elution with benzene:methanol (99:1, v/v) afforded a gum which showed only a fraction (≡ 4 mg.) of the initial absorption at 282 mμ. Further attempts at purification failed.

Tubes 37–49: the neutral fraction (40 mg.) showed $\lambda_{max.}$ 280, ~290 mμ (≡ 29 mg. of grisan-3:4′-diol) and was chromatographed as described above. Fractions showing $\lambda_{max.}$ 282 mμ were combined and sublimed at 150–160°/0·03 mm. pressure; the sublimate was treated with ether, furnishing a solid which was crystallized from benzene, yielding prisms (1 mg.), m.p. 171–186° of a mixture of α- and β-grisan-3:4′-diols in approximately equal proportions as shown by the infrared spectrum.

Compound dd-(II; R = OMe). No differences were observed between the griseofulvin-treated and control extracts in the decay experiments.

DISCUSSION

The spectrophotometric methods of analysis outlined above proved satisfactory in practice; on plant-tissue extracts containing 1–20 mg. of the compounds they were accurate to ± 1 mg. (Crowdy *et al.* 1955) considered adequate for work of this type. Estimations on treating solutions were probably rather less accurate, as the elaborate series of control experiments performed with griseofulvin (Crowdy *et al.* 1955) was not carried out.

The griseofulvin *ld* diastereoisomer, dechlorogriseofulvin, 7-chloro-6:2′-diethoxy-4-methoxy-6′-methylgris-2′-en-3:4′-diol. (V) and the alcohol (III; R = H) were recovered, unchanged, from both shoots and roots of treated plants; and the propyl and butyl homologues of griseofulvin and the diol (III; R = OH) were recovered from the roots. In addition there was strong presumptive evidence that the last three compounds, and also 7-chloro-4:6:4′-trimethoxy-6′-methylgris-3′-en-3:2′-dione, were present unchanged in the shoots; and that the amine (II; R = NH_2) existed unchanged in both roots and shoots. The trione (II; R = OH) and grisan-3:4′-dione were absent from both roots and shoots.

In the 3-day-uptake experiments described in this paper it was not expected, by analogy with griseofulvin (Crowdy *et al.* 1955), that metabolites of the compounds studied would be detected or isolated. This expectation proved to be correct for the compounds more closely related to griseofulvin, namely the *ld*-diastereoisomer, the dechloro analogue and three homologues of griseofulvin and also for the alcohols (III; R = H and OH). On the

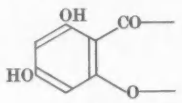
other hand, both compound (IV) and grisan-3:4′-dione gave rise during 3 days to relatively stable metabolites in amounts permitting their detection and identification. With the compound (IV) additional metabolites were detected, though not identified, in both roots and treating solution. Of the metabolites identified 3-chloro-2-hydroxy-4:6-dimethoxybenzoic acid may be regarded as an oxidation product of compound (IV), whereas the grisan-3:4′-diols and 4′-hydroxygrisan-3-ones are reduction products of grisan-3:4′-dione. Although it was not possible to estimate accurately the relative amounts of the α- and β-grisan-3:4′-diols present in the whole plant system, including the treating solution, the diol mixture actually isolated from the various sites (some 54% of the total, estimated spectrophotometrically) was shown to contain not less than 46% of the β (less stable) isomer. Thus the proportion of the β-diol present in the entire system was at least 25%.

Root extracts of plants treated with the amine (II; R = NH_2) and with the butyl homologue of griseofulvin contained traces of an acidic substance believed to be the trione (II; R = OH); a trace of an unidentified neutral substance was also detected in roots treated with the amine. The trione (II; R = OH) is unstable in bean roots, none having been recovered from the roots of treated plants, and it follows that the acidic substance was probably produced by hydrolysis of the amine (II; R = NH_2) and butyl homologue during maceration and extraction.

With these two compounds and with grisan-3:4′-dione the final pH of the treating solution was abnormally low (Table 2), but there was no correlation between low pH and the production in the treating solution of acidic metabolites detectable by the spectrophotometric techniques employed. Acidic metabolites were only detected in the residual solution from plants treated with the dechloro analogue of griseofulvin and with (IV). In the former case the acidic substance was believed to be the corresponding trione (VI; R = OH).

No metabolites of griseofulvin were detected in the shoots of treated plants following the 3-day-uptake experiment (Crowdy *et al.* 1955). Griseofulvin is nevertheless slowly broken down in the shoots of broad bean (Crowdy *et al.* 1956), and the shoot-extracts from two decay experiments, in which griseofulvin was estimated chemically, were examined for the presence of metabolites. An acidic fraction, absent from the control, with strong absorption peaks at 285 and 320 mμ, showing a characteristic shift to 293 and 345 mμ in alkaline solution, was obtained in both experiments. A similar acidic fraction, absent from the control, and showing identical ultraviolet-absorption characteristics, was obtained from the shoots of plants treated for 3 days with the amine (II; R = NH_2; Table 4). Traces of acidic substances with a similar absorption spectrum in ethanol were also found in the shoots of plants treated with the dechloro analogue, the homologue (V), the *ld*-diastereoisomer and the diol (III; R = OH). The acidic substance, first detected in the griseofulvin-decay experiments, was for some time believed to be a metabolite of griseofulvin; the possibility that it might be a metabolite common to the breakdown of all the above chlorine-containing griseofulvin relatives was also considered. The observed absorption shift in alkali is characteristic of the 4:6-dihydroxycoumaranone chromophore (VIII) (Duncanson *et al.* 1957). The substance was labile, being particularly sensitive to atmospheric oxidation, and although some purification was effected by chromatographic and distillation techniques it remained unidentified. The halogen contents of purified fractions were nevertheless no greater than those of controls from untreated plants.

An acidic fraction with identical ultraviolet-absorption maxima was eventually obtained from the shoot-extract control to the large-scale grisan-3:4′-dione experiment (*B*); the corresponding fraction from the treated plants showed an identical absorption. The substance must therefore be a normal plant constituent, a conclusion in agreement with the halogen determinations; it seems unlikely that the griseofulvin molecule would lose halogen without disruption of the aromatic ring (Wright & Grove, 1957). The absence of the acidic substance from the appropriate controls in all the cases listed in Table 4 is most striking; the most likely explanation seems to be that the substance, a normal plant constituent, is produced in much greater quantity in shoots treated with griseofulvin relatives. The partial structure (VIII), suggested by the ultraviolet-absorption data, is common to many known plant constituents.



(VIII)

The spectrophotometric techniques employed in this work are well suited to the routine estimation of the compounds examined; they are not so well suited to metabolic studies which could more conveniently be carried out on labelled compounds with tracer techniques.

SUMMARY

1. Analytical methods, similar to those used for griseofulvin (Crowdy *et al.* 1955), are described for the estimation in bean tissues and the treating

solutions of eleven compounds chemically related to griseofulvin. The compounds studied comprised a diastereoisomer of griseofulvin, dechlorogriseofulvin, three homologues of griseofulvin, 7-chloro-4:6:4′-trimethoxy-6′-methylgris-3′-en-3:2′-dione, the parent acidic trione, its basic amino derivative, two neutral alcoholic reduction products of the trione, and grisan-3:4′-dione.

2. The compounds were recovered and characterized chemically in the majority of cases where the analytical methods indicated their presence in the roots and shoots of treated plants. 3-Chloro-2-hydroxy-4:6-dimethoxybenzoic acid, a metabolite of 7-chloro-4:6:4′-trimethoxy-6′-methylgris-3′-en-3:2′-dione and mixtures of 4′-hydroxygrisan-3-ones and optically active grisan-3:4′-diols, metabolites of grisan-3:4′-dione, were also recovered and identified. Other metabolites of these two compounds, and a substance, increased production of which is associated with the degradation of certain griseofulvin relatives in shoot tissue, were detected but not identified.

We are indebted to Mrs A. Borrow and to Messrs D. Gardner and P. W. Jeffs for technical assistance and to Dr L. A. Duncanson for the infrared spectra.

REFERENCES

Abbot, M. T. J., Grove, J. F. & McCloskey, P. (1958). *J. chem. Soc.* p. 1699.
Cahn, R. S., Ingold, C. K. & Prelog, V. (1956). *Experientia*, **12**, 81.
Crowdy, S. H., Gardner, D., Grove, J. F. & Pramer, D. (1955). *J. exp. Bot.* **6**, 371.
Crowdy, S. H., Grove, J. F., Hemming, H. G. & Robinson, K. C. (1956). *J. exp. Bot.* **7**, 42.
Crowdy, S. H., Grove, J. F. & McCloskey, P. (1959). *Biochem. J.* **72**, 241.
Duncanson, L. A., Grove, J. F. & Jeffs, P. W. (1958). *J. chem. Soc.* p. 2929.
Duncanson, L. A., Grove, J. F., MacMillan, J. & Mulholland, T. P. C. (1957). *J. chem. Soc.* p. 3555.
Grove, J. F., MacMillan, J., Mulholland, T. P. C. & Rogers, M. A. T. (1952*a*). *J. chem. Soc.* p. 3949.
Grove, J. F., MacMillan, J., Mulholland, T. P. C. & Rogers, M. A. T. (1952*b*). *J. chem. Soc.* p. 3977.
Jones, D. R. & Wignall, J. (1955). *Nature, Lond.*, **175**, 207.
McCloskey, P. (1958). *J. chem. Soc.* p. 4732.
MacMillan, J. (1953). *J. chem. Soc.* p. 1697.
MacMillan, J. (1959). *J. chem. Soc.* p. 1823.
Morton, R. A. & Stubbs, A. L. (1946). *Analyst*, **71**, 348.
Williamson, B. & Craig, L. C. (1947). *J. biol. Chem.* **168**, 687.
Wright, J. M. & Grove, J. F. (1957). *Ann. appl. Biol.* **45**, 36

The Translocation of Antibiotics in Higher Plants

4. SYSTEMIC FUNGICIDAL ACTIVITY AND CHEMICAL STRUCTURE IN GRISEOFULVIN RELATIVES

BY S. H. CROWDY, JOHN FREDERICK GROVE AND P. McCLOSKEY
Imperial Chemical Industries Ltd., Akers Research Laboratories, Welwyn, Herts

(*Received* 29 *September* 1958)

Griseofulvin causes a characteristic distortion ('curling') of the hyphae of most fungi with chitinous cell walls, and the capacity to produce this hyphal distortion seems to be specific to griseofulvin and its near relatives (J. F. Grove, unpublished work). By suitable modification of the molecule it is possible to produce a range of compounds in which the antifungal activity is varied widely. With suitable biological testing it is possible to show how far the systemic fungicidal activity of these compounds is related to their ability to induce hyphal distortion. In tracing this relationship systemic fungicidal activity must be assessed in relation to the amount of the compound actually present in the plant tissues. To this end analytical methods, similar to those used for griseofulvin (Crowdy, Gardner, Grove & Pramer, 1955), were worked out (Crowdy, Green, Grove, McCloskey & Morrison, 1959) for estimating the griseofulvin relatives in plant tissue and in the treating solutions. Balance sheets were then drawn up relating the amount of material actually entering the plant to that found in the roots and shoots.

EXPERIMENTAL

Analytical methods have been described in detail in the preceding paper (Crowdy *et al.* 1959).

Antifungal tests. The antifungal activity *in vitro* was assessed against germinating spores of *Botrytis allii* (Brian, Curtis & Hemming, 1946). Concentrations of griseofulvin causing hyphal 'curling' and stunting of *Alternaria solani* are similar to those having a like effect on *B. allii*.

Details of the test for systemic fungicidal activity, measured by the reduction of leaf infection of tomato by *A. solani* after treatment of the roots with 0·05% dispersions of the compounds, have been given by Brian, Wright, Stubbs & Way (1951), and Stubbs (1952). Reproducibility in this test was not high and the results have been graded for convenience on an arbitrary scale (Table 1).

Compounds giving 0–49% control of disease were considered unlikely to be of importance in practice and were

arbitrarily graded 'inactive'. Phytotoxic damage was observed only when the plants were treated with grisan-3:4′-dione.

The compounds studied are listed in Table 2.

The determination of the water solubilities and oil–water partition coefficients will be described elsewhere (J. R. Bartels-Keith & J. F. Grove, unpublished work). For convenience in measuring the partition coefficients of the higher homologues of griseofulvin, hexane was chosen as the model 'lipid' material. Partition coefficients for olive oil–water were approx. 30 times those given in Table 2 for hexane–water.

RESULTS

Relation between antifungal and systemic fungicidal activities in vitro

The results of the tests for systemic fungicidal activity are compared in Table 2 with the lowest concentration causing 'curling' of *B. allii* (activity *in vitro*), and there is clearly no direct correlation between the two tests. The griseofulvin *ld*-diastereoisomer is inactive *in vitro* and shows no systemic fungicidal properties. But the dechloro analogue (VI) shows systemic fungicidal properties, although its activity *in vitro* is low; on the other hand, 7-chloro-4:6-dimethoxy-6′-methylgris-2′-en-3:4′-dione (II; R = H), which is structurally similar to griseofulvin and has marked activity *in vitro* shows no systemic fungicidal activity. The enol ether (IV; R = OMe), isomeric with griseofulvin, has more than ten times the activity *in vitro* of the dechloro analogue (VI), but no systemic fungicidal properties. By contrast, the acidic trione (II; R = OH) and grisan-3:4′-dione (I) are inactive *in vitro* but show weak systemic fungicidal activity.

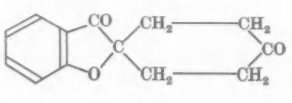
The amine (II; R = NH_2) has no *in vitro* or systemic fungicidal activity; in the work on the translocation of these derivatives it was of interest to compare the behaviour of the neutral griseofulvin (II; R = OMe) with the acidic trione (II; R = OH) and the basic amine (II; R = NH_2).

Some results with homologues both of griseofulvin and of (IV; R = OMe) are contained in section II of Table 2. In the griseofulvin series (II; R = O-*n*-alkyl) there is an increase in activity *in vitro* up to a maximum at R = OPr^n followed by a 'cut-off' at R = OBu^n; systemic fungicidal properties are found when R = OEt but higher homologues are inactive. However, the griseofulvin homologue 7-chloro-6:2′-diethoxy-4-methoxy-6′-methylgris-2′-en-3:4′-dione (V) isomeric with (II; R = OPr^n), but in which the two additional carbon atoms are distributed between two alkoxyl substituents, shows pronounced systemic fungicidal

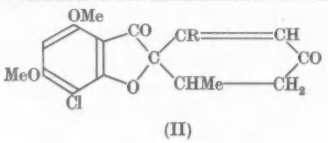
Table 1. *Relationship between control by chemical compounds of* A. solani *infection of tomato and grading of systemic fungicidal activity*

Tomato plants, 6 in. high, grown in sand culture were treated with 0·05% dispersions of the compounds. On the fifth day plants were inoculated with *A. solani* spores and on the eighth day the number of lesions on the lower five leaves of each plant was counted. The total number of lesions on treated plants (3 replicates) expressed as a percentage of those on control plants gave a measure of the control of disease, which was more conveniently expressed by the grading of systemic fungicidal activity.

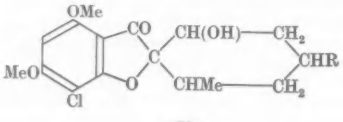
% Control of disease	Grading
100–95	+ + +
94–85	+ +
84–75	+
74–50	±
49–0	−



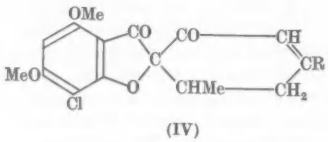
(I)



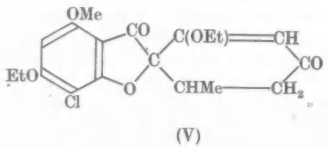
(II)



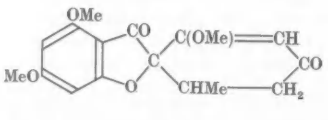
(III)



(IV)



(V)



(VI)

Table 2. *Comparison of* in vitro *and systemic fungicidal activities of griseofulvin and related compounds*

Compound	Structure	Water-solubility (μM)	Hexane–water partition coeff.	Least concn. causing curling of *B. allii* (μM)	Systemic fungicidal activity in tomato
I. Griseofulvin relatives					
dd-7-Chloro-4:6:2′-trimethoxy-6′-methylgris-2′-en-3:4′-dione (griseofulvin)	(II; R = OMe)	25	0·80	0·28	+ + +
ld-7-Chloro-4:6:2′-trimethoxy-6′-methylgris-2′-en-3:4′-dione	(II; R = OMe)	32	1·80	Inactive	–
4:6:2′-Trimethoxy-6′-methylgris-2′-en-3:4′-dione	(VI)	217	0·36	19	+
7-Chloro-4:6-dimethoxy-6′-methylgris-2′-en-3:4′-dione	(II; R = H)	650	4·19	2·5	–
7-Chloro-4:6:4′-trimethoxy-6′-methylgris-3′-en-3:2′-dione	(IV; R = OMe)	37	1·37	1·3	–
Tetra-acetyl-2′-glucosyloxy-7-chloro-4:6-dimethoxy-6′-methylgris-2′-en-3:4′-dione	(II; R = $OC_6H_7O_5Ac_4$)	120	0·0675	75	–
7-Chloro-4′:6′-dihydroxy-4:6-dimethoxy-2′-methylgrisan-3-one	(III; R = OH)	4580	0·0015	18	–
7-Chloro-6′-hydroxy-4:6-dimethoxy-2′-methylgrisan-3-one	(III; R = H)	214	0·39	76·5	–
7-Chloro-4:6-dimethoxy-2′-methylgrisan-3:4′:6′-trione	(II; R = OH)	pH-dependent	—	Inactive	±
2′-Amino-7-chloro-4:6-dimethoxy-6′-methylgris-2′-en-3:4′-dione	(II; R = NH_2)	pH-dependent	—	Inactive	–
Grisan-3:4′-dione	(I)	6940	3·59	Inactive	±*
II. Homologues of griseofulvin and of (IV; R = OMe)					
7-Chloro-2′-ethoxy-4:6-dimethoxy-6′-methylgris-2′-en-3:4′-dione	(II; R = OEt)	12	2·28	0·14	+ +
7-Chloro-4:6-dimethoxy-6′-methyl-2′-*n*-propoxygris-2′-en-3:4′-dione	(II; R = OPr^n)	29	9·73	0·013	–
2′-*n*-Butoxy-7-chloro-4:6-dimethoxy-6′-methylgris-2′-en-3:4′-dione	(II; R = OBu^n)	19	18·7	0·013	–
7-Chloro-6:2′-diethoxy-4-methoxy-6′-methylgris-2′-en-3:4′-dione	(V)	17	9·47	0·13	+ +
7-Chloro-4′-ethoxy-4:6-dimethoxy-6′-methylgris-3′-en-3:2′-dione	(IV; R = OEt)	25	3·70	1·1	–
7-Chloro-4:6-dimethoxy-6′-methyl-4′-*n*-propoxygris-3′-en-3:2′-dione	(IV; R = OPr^n)	5·8	23·7	1·0	–
4′-*n*-Butoxy-7-chloro-4:6-dimethoxy-6′-methylgris-3′-en-3:2′-dione	(IV; R = OBu^n)	3·8	52·0	1·0	–
7-Chloro-4′-*n*-hexyloxy-4:6-dimethoxy-6′-methylgris-3′-en-3:2′-dione	(IV; R = O-*n*-C_6H_{13})	2·1	640	0·95	–

* Phytotoxicity symptoms.

activity. In the series (IV; R = O-*n*-alkyl), all homologues of (IV; R = OMe) are inactive in the systemic fungicidal test, although in this series also the higher homologues show slightly increased activity *in vitro* up to a 'cut-off' at R = O-*n*-C_6H_{13}.

Preliminary results obtained by Stokes (1954) on the translocation of griseofulvin in wheat suggested that the amount of griseofulvin in the guttation fluid was a function of the water transpired. It followed that water-solubility might be an important factor in translocation, and some derivatives with a higher water-solubility than griseofulvin, but of lower activity *in vitro*, were investigated. The diol (III; R = OH) has activity *in vitro* comparable with that of the dechloro analogue (VI) and much greater water-solubility, but had no systemic fungicidal activity. The alcohol (III; R = H) and the tetra-acetylglucoside (II; R = $OC_6H_7O_5Ac_4$) were also inactive as systemic fungicides: the former compound has the same water-solubility and hexane–water partition coefficient as (VI) but lower activity *in vitro*.

On the results of these tests the compounds can be placed in four categories:

Type *A* showed activity in both tests, e.g. the two homologues (II; R = OEt) and (V) of griseofulvin and the dechloro analogue (VI). These compounds were closely related chemically to griseofulvin.

Type *B*. Some compounds with activity *in vitro* similar to or greater than that of griseofulvin, e.g. 7-chloro-4:6:4′-trimethoxy-6′-methylgris-3′-en-3:2′-dione (IV; R = OMe) and the propyl and butyl homologues of griseofulvin, had no systemic fungicidal activity. The alcohols (III; R = H and OH) bore the same relationship to the dechloro analogue (VI).

Type *C* showed weak systemic fungicidal properties although inactive *in vitro*, e.g. the trione (II; R = OH) and grisan-3:4′-dione (I).

Type *D* were inactive in both tests, e.g. the amine (II; R = NH_2) and the griseofulvin *ld*-diastereoisomer.

It was unlikely that any understanding of these results would be achieved by further empirical testing: one obvious conclusion was that the *B. allii* test provided no indication whether a particular griseofulvin relative would be active as a systemic fungicide. The weight-balance experiments described in the next section were designed to throw some light on these inconsistencies.

Uptake and distribution in the plant

The plant selected for study was the broad bean with which the results of tests for systemic fungicidal activity are qualitatively the same as those obtained with tomato (Table 2). The analytical methods (Crowdy *et al.* 1959) were laborious and it was possible to study only a small number of compounds. The compounds were selected to provide answers to the following questions:

(1) Are those griseofulvin relatives which are active both *in vitro* and as systemic fungicides translocated unchanged? Or is systemic fungicidal activity due to the formation of some metabolite, or to other causes?

(2) Why do certain griseofulvin derivatives show systemic fungicidal properties although inactive *in vitro*?

(3) Are griseofulvin relatives active *in vitro* but inactive as systemic fungicides not taken up by the root system? Or are they broken down in the plant tissues before reaching the shoots?

The results of weight-balance experiments (Crowdy *et al.* 1959) in which the amount of chemical taken up by the plants has been compared with that actually found in the roots and shoots are set out in Table 3. To simplify the presentation of the data a standardized treatment of 100 mg. has been selected and the remaining figures have been adjusted in proportion to this standard. In most cases the adjustment is small since the original weight of compound added to the treating solution was about 125 mg.: with the propyl and butyl homologues (25 mg.) the adjusted figures may be subject to error. Two sets of figures are given for the uptake. The first, experimental uptake, is the difference between the amount added to the treating solution (100 mg.) and the amount recovered at the end of treatment. It is compared with the sum of the amounts found in the roots and shoots; the difference includes all analytical errors but gives some idea of the amount lost by decay. The latter, together with the amounts present in shoots and roots, is also expressed in the last three columns of Table 3, as a percentage of the experimental uptake. In calculating these figures traces (<0·5 mg.) of compounds present in the shoots have been taken arbitrarily as zero. The percentage lost by decay is apparently lowest with griseofulvin; this possibly reflects the somewhat greater precision of the method employed for estimating griseofulvin in the treating solution when compared with the corresponding methods of estimation for the griseofulvin relatives (Crowdy *et al.* 1959).

The calculated uptake is a hypothetical figure based on the behaviour of griseofulvin as described by Crowdy, Grove, Hemming & Robinson (1956): the method of calculation has been described in the preceding paper. With the exception of 7-chloro-4:6:4′-trimethoxy-6′-methylgris-3′-en-3:2′-dione (IV; R = OMe) and grisan-3:4′-dione, where metabolites were excreted in quantity into the treating solution, agreement with the experimental estimation of uptake is reasonably good. This is surprising

in view of the very wide variation in oil–water partition coefficients among the compounds studied. With griseofulvin some accumulation in the roots, attributed to partition into a lipid phase in the tissues, took place in the absence of transpiration; and uptake arising from accumulation of this type was expected to be of increased importance with the more lipid-soluble griseofulvin homologues. The general coincidence of the figures for experimental and calculated uptakes for these compounds may be attributed to a reduction in the amounts removed from the roots on the transpiration stream under the non-equilibrium conditions which inevitably operate in these short-term experiments. The contribution to total uptake made by partition into the root lipids is also reflected in the experimentally determined figures for the uptakes of the water-soluble alcohols (III; R = H and OH); these fall somewhat below the calculated values. Nevertheless, removal of the compounds from the roots on the transpiration stream remains the most important factor determining uptake.

Compounds are discussed according to the classification set out above. In Table 4 the amount present in the shoots has been divided by the fresh weight of shoots (Crowdy *et al.* 1959) giving the concentration: the latter is compared with the lowest concentration active *in vitro*.

Type A. Nearly all the griseofulvin taken up during three days was accounted for in the roots and shoots and no metabolites could be detected (Crowdy *et al.* 1955). Breakdown did, however, take place and in the 6·5- and 14-day decay experiments (Crowdy *et al.* 1956) 80 and 92 % respectively of the griseofulvin taken up was metabolized. Although no metabolites were isolated, the

Table 3. *Location of compounds in broad-bean plants after* 3 *days' treatment*

The data refer to a standard treatment with 100 mg. of compound (see text). 'Trace' is equivalent to 'less than 0·5 mg.'

Compound	Expt.	Type	Uptake (mg.) Exptl.	Uptake (mg.) Calc.	Distribution in plant (mg.) Roots	Shoots	Missing (decayed)	Distribution (% of exptl. uptake) Roots	Shoots	Missing (decayed)
dd-(II; R = OMe)		*A*	33	33	16	9	8	48	27	25
(VI)		*A*	35	22	7	8	20	20	23	57
(V)		*A*	40	37	23	3	14	58	8	34
(IV; R = OMe)		*B*	54*	25	0†	1	53	0	2	98
(II; R = OPr^n)		*B*	32	40	16	1	15	50	3	47
(II; R = OBu^n)	B	*B*	32	36	20	Trace	12	63	0	37
(III; R = OH)		*B*	24	37	8	Trace	16	33	0	67
(III; R = H)		*B*	19	36	7	4	8	37	21	42
(II; R = OH)	A	*C*	45	33	0	0	45	0	0	100
	B		73	77	0	0	73	0	0	100
(I)	A	*C*	90*	31	0†	0†	90	0	0	100
	B		98*	37	0†‡	0†‡	98‡	0	0	100
ld-(II; R = OMe)		*D*	35	41	11	3	21	31	9	60
(II; R = NH_2)		*D*	42	33	3†	Trace	39	7	0	93

* Metabolites found in residue of treating solution. † Metabolites found in tissue extracts.
‡ Total metabolites (mg.): roots (10), shoots (12), treating solution (31); 7, 9 and 23 mg. respectively were grisan-3:4′-diols.

Table 4. *Comparison of concentrations present in bean-shoot tissue with activity* in vitro

'Grading' refers to the systemic fungicidal activity.

Compound	Type	Grading	Concn. in shoots (μmoles/kg. fresh wt.)	Concn. active *in vitro* (μM)
dd-(II; R = OMe)	*A*	+ + +	51	0·28
(V)	*A*	+ +	24	0·13
(VI)	*A*	+	36·5	19
(III; R = H)	*B*	–	39	75
(IV; R = OMe)	*B*	–	4·4*	1·3
(II; R = OPr^n)	*B*	–	0·8*	0·013
(II; R = OBu^n)	*B*	–	—*	0·013
(III; R = OH)	*B*	–	—*	18

* Compound not formally identified in shoot tissue.

presence of a substance which seemed to be associated with the breakdown of griseofulvin relatives in shoot tissue was detected; this substance had no activity *in vitro*.

Assuming an even distribution, the concentration of griseofulvin present after 3 days (Table 4) was 51 μmoles/kg. (fresh wt.) of shoot tissue, a figure considerably in excess of that (0·28 μM) having activity *in vitro*. In practice an even distribution is unlikely, but with griseofulvin wide deviations from the mean are clearly permissible.

The dechloro analogue (VI) was also translocated unchanged and appreciable quantities were present in the roots and shoots; breakdown was more rapid than with griseofulvin but no metabolites were detected. The mean concentration in shoot tissue, 36·5 μmoles/kg. (fresh wt.) was about double the concentration active *in vitro*. 7-Chloro-6:2′-diethoxy-4-methoxy-6′-methylgris-2′-en-3:4′-dione (V) tended to accumulate in the roots and only 8 % of that taken up was present in the shoots; this reduction, compared with griseofulvin (leading to a concentration of 24 μmoles/kg. fresh wt.), was compensated by the increased activity *in vitro*.

All three compounds of type *A* reached the shoots unchanged and were present there in a concentration adequate for control of the pathogen. These results are therefore readily correlated with the results of the test for systemic fungicidal activity.

Type B. The tendency for griseofulvin homologues (II; R = O-alkyl) to accumulate in the roots, already noticed in connexion with 7-chloro-6:2′-diethoxy-4-methoxy-6′-methylgris-2′-en-3:4′-dione, was more apparent with the propyl and butyl homologues. Only 1 % of the propyl homologue taken (3 % of that actually taken up by the plants) reached the shoots and only a trace of the butyl homologue could be detected there. The rates of decomposition for these compounds in the plant were not significantly greater than those for compounds of type *A*, and no metabolites were detected: their low water-solubility coupled with a high oil–water partition coefficient might be expected to operate against their movement in the transpiration stream and be the cause of their absence from the shoots and their accumulation in the roots (Table 3). It may be noted that 7-chloro-6:2′-diethoxy-4-methoxy-6′-methylgris-2′-en-3:4′-dione (V) (type *A*) has a slightly lower oil–water partition coefficient than the isomeric propyl homologue has. The low concentration of the highly active propyl homologue present in shoot tissue was nevertheless in excess of that required for activity *in vitro*. Quantitatively, therefore, the correlation with the systemic fungicidal activity grading is not so satisfactory. These calculations, however, assume an even distribution of the compound, a distribution which is unlikely in practice. Moreover, as with griseofulvin (Crowdy *et al.* 1956), the compounds may be retained more strongly in the roots of tomato.

The water-soluble diol (III; R = OH) was taken up by the plants and was recovered from the roots, but none was present in the shoots; the high overall loss (67 % of that taken up) suggested that the compound was broken down more rapidly than were the griseofulvin homologues: no metabolites were detected, however. A similar situation, in more exaggerated form, was found with 7-chloro-4:6:4′-trimethoxy-6′-methylgris-3′-en-3:2′-dione (IV; R = OMe), which could not be detected in the roots; 98 % of that taken up was unaccounted for in the plant tissues. Two metabolites, 3-chloro-2-hydroxy-4:6-dimethoxybenzoic acid and an unidentified acid, m.p. 284–286°, were isolated from the roots, and other metabolites, both neutral and acidic, were detected in the plant tissues and the treating solution. Quantitatively the concentration believed to be present in shoot tissue was again in excess of that active *in vitro*; the ratio of the two concentrations was, however, of a different order from that found with griseofulvin. The alcohol (III; R = H) was translocated, unchanged, and was more stable in plant tissue than was the related diol (III; R = OH). The concentration in the shoots was similar to that attained by the dechloro analogue (VI), which had similar solubility properties; however, this concentration was lower (Table 4) than that causing activity *in vitro*, in agreement with the systemic fungicidal activity grading.

Thus with compounds of type *B*, often highly active *in vitro*, there is a correlation between the (negative) results of the tests for systemic fungicidal activity and the concentrations (very small or zero or below that active *in vitro*) of the compounds actually present in the shoots. It seems probable that these compounds fail to reach the shoots either because they are broken down rapidly in the roots or because their solubility properties operate against translocation from root to shoot.

Type C. Although the trione (II; R = OH) was taken up from the treating solution, none could be detected in the roots or shoots. Moreover, despite a careful search and the very high uptake induced in Expt. B no metabolites were detected: in particular, the acidic substance which was associated with the breakdown of griseofulvin relatives in shoot tissue was absent. This last observation suggests that the breakdown of the trione takes place in the roots and is complete, and the weak systemic fungicidal activity found in plants treated with the trione must therefore be due to secondary, indirect, causes. A similar explanation no doubt accounts for the weak systemic fungicidal activity of grisan-3:4′-dione which was observed to

cause retardation of growth of the plants. No grisan-3:4′-dione was present in the roots or shoots and only very little remained in the treating solution. Instead, quantities of reduction products, stereoisomeric grisan-3:4′-diols, were found in treating solution, roots and shoots, and smaller amounts of the intermediate 4′-hydroxygrisan-3-ones were probably present in the shoots.

Both the grisan-3:4′-diols and 4′-hydroxygrisan-3-ones, like the dione, were inactive *in vitro* at 500 μM against *B. allii*. The total weight of these and other unidentified metabolites estimated accounted for only half of the grisandione taken (Table 3): the remainder must have been modified further either by this or alternative routes.

Type D. The amine (II; R = NH_2) was taken up from the treating solution, but very little was found in the roots and only a trace in the shoots. The latter contained some of the acidic substance associated with the breakdown of griseofulvin relatives, suggesting that the amine was not broken down so readily in the roots as was the trione (II; R = OH). A metabolite was nevertheless detected in the roots and the general picture was similar to that presented by (IV; R = OMe) of type *B*.

The griseofulvin *ld*-diastereoisomer was taken up and translocated to the shoots, unchanged; there was some evidence that it was broken down more readily than griseofulvin.

DISCUSSION

The advantages gained by introducing systemic fungicides into plants through the root system have been discussed by Brian (1952). Systemic fungicides applied in this manner may act directly on the pathogen or indirectly in a variety of ways. It has generally been assumed that compounds active both *in vitro* and as systemic fungicides are translocated from roots to shoots unchanged and act directly on the pathogen. This hypothesis, originally advanced for griseofulvin by Brian *et al.* (1951) and by Stokes (1954), has been shown to be true by the present studies on translocation (Crowdy *et al.* 1955) and by the work of Aytoun (1956). It has now been shown to be true for griseofulvin relatives of type *A*; it is reinforced by the absence of systemic fungicidal properties in the griseofulvin *ld*-diastereoisomer which is inactive *in vitro* but, like griseofulvin, is translocated, unchanged.

Compounds inactive *in vitro* but active as systemic fungicides must clearly function by an indirect mechanism and several hypotheses have been advanced to explain this mode of action. Some compounds are thought to be modified chemically within the plant with formation of a stable antifungal substance which is translocated; such a mechanism has been proposed by van Raalte, Sijpesteijn, van der Kerk, Oort & Pluygers (1955) to explain the activity of certain derivatives of dimethyldithiocarbamic acid. Others may owe activity to their phytotoxic properties: damaged plants pass through a stage in which they are more resistant than normal towards certain specific infections (Keyworth & Dimond, 1952). A third group of non-phytotoxic compounds would appear to alter the resistance of the host plant to the pathogen either, for example, by stimulating production of weakly antifungal phenolic and other substances normally present in the tissues or more obliquely by processes which may also result in a formative action on the plant. The plant-growth regulators investigated by Davis & Dimond (1953) and certain benzothiazole derivatives (Dimond & Davis, 1953) come into this last category.

The systemic fungicidal activity of the trione (II; R = OH) and grisan-3:4′-dione must be due to an indirect mechanism, not necessarily the same for both compounds. No antifungal metabolites of the two compounds were found in the plant tissues; neither did there appear to be a significantly greater concentration of phenolic compounds in the treated plants compared with the controls. Both compounds underwent complete metabolic transformation in the roots, and resistance to the pathogen must be associated in some way with this unusual metabolic activity. Phytotoxic symptoms were observed only in plants treated with grisan-3:4′-dione or with its metabolites, (+)-α-grisan-3:4′-diol and α-4′-hydroxygrisan-3-one.

All compounds, whether neutral, acidic or basic, were taken up by the plant roots. This was of interest as the trione (II; R = OH) and the amine (II; R = NH_2) were present largely as the anion and cation respectively at the pH of the treating solution. With the exception of those cases where metabolites were rapidly excreted into the treating solution, the amount of the compound taken up was related to the water transpired.

Correlation between the translocation studies and the results of tests for systemic fungicidal activity was good. A systemic fungicide based chemically on the griseofulvin structure and acting directly on the pathogen after application through the roots of the host plant should have the following properties:

(*a*) *High activity* in vitro.

(*b*) *Solubility properties favouring translocation from root to shoot.* Compounds with a wide range of water-solubility and oil–water partition coefficient were deliberately chosen for study, but, since many were rapidly degraded by the roots, straightforward solubility effects were masked. Translocation in the transpiration stream should be favoured

by high water-solubility coupled with a low oil–water partition coefficient. Physical properties such as solubility contribute to the activity *in vitro* of griseofulvin relatives, and within each closely similar group of compounds, such as a homologous series, there is an optimum high oil–water partition coefficient. For systemic fungicidal activity, therefore, there will be an optimum oil–water partition coefficient and a minimum water-solubility, both related to the *in vitro* activity. With broad bean, compounds with a hexane–water partition coefficient greater than 6 tend to be retained in the roots; a value of 0·8 is satisfactory but 0·3 may be advantageous.

Increasing the lipid-solubility of griseofulvin by extending the chain-length of the 2′-alkoxyl substituent gives compounds with enhanced activity *in vitro*, but these compounds accumulate in the root lipids and are not translocated so readily to the shoot. A water-solubility of 20 μM is adequate for compounds with activity *in vitro* of the same order as that of griseofulvin.

(c) *Chemical stability in root tissue.* This property is difficult to predict and no definite pattern emerges from the present study. Roots are a site of vigorous metabolic activity and rapidly degrade many structures of the griseofulvin type. Surprisingly the most stable arrangement investigated was the 2′-alkoxygris-2′-en-3:4′-dione structure found in griseofulvin itself. The contrast with the isomeric 4′-alkoxygris-3′-en-3:2′-dione structure, which was rapidly degraded to give *inter alia* 3-chloro-2-hydroxy-4:6-dimethoxybenzoic acid was astonishing. 3-Chloro-2-hydroxy-4:6-dimethoxybenzoic acid is frequently a product of the oxidative chemical degradation of griseofulvin derivatives. The neutral metabolite, $\lambda_{max.}$ 291 mμ (Crowdy *et al.* 1959), but not the phenolic acid, m.p. 284–286°, could be an intermediate in its formation in the bean root. That 3-chloro-2-hydroxy-4:6-dimethoxybenzoic acid is not isolated from other compounds, e.g. the trione (II; R = OH), the amine (II; R = NH_2) and the diol (II; R = OH), where extensive breakdown takes place in the roots, only serves to emphasize the multiplicity of degradative mechanisms available. A further illustration of this is provided by grisan-3:4′-dione, where reduction to stereoisomeric (+)-grisan-3:4′-diols via the intermediate 4′-hydroxygrisan-3-ones occurs. In this connexion it is perhaps significant that flavonols and the corresponding anthocyanidins and catechins, e.g. quercitin, cyanidin and *epi*catechin, frequently occur together in the same plants. Grisan-3-ol corresponds to the intermediate oxidation state, cyanidin pseudo-base. In the 2′-alkoxy-4:6-dimethoxygris-2′-en-3:4′-dione structure the carbonyl group in the 3 position is sterically hindered by the 4-methoxyl substituent, while the 4′-carbonyl group is conjugated with an ethylenic double bond, and reduction of the carbonyl groups does not take place.

Further work may reveal exceptions to these conclusions, which are based on results obtained with only a small number of compounds; moreover, empirical rules which apply to broad bean or tomato and *A. solani* will not necessarily hold for other plants and pathogens. At the same time it is considered that some progress has been made in relating systemic fungicidal activity and structure among griseofulvin relatives.

SUMMARY

1. No direct correlation was found between *in vitro* and systemic fungicidal activities among griseofulvin relatives. In an attempt to elucidate the reasons for this disparity, the translocation and breakdown of a selected group of griseofulvin relatives in broad bean was studied by the methods outlined in the preceding paper. All the compounds, whether neutral, acidic or basic, were taken up through the root system.

2. Compounds active *in vitro* and as systemic fungicides were closely related to griseofulvin in structure, were translocated unchanged and acted directly against the pathogen.

3. Compounds active *in vitro* but inactive as systemic fungicides did not attain the appropriate minimal concentration in the shoots. This was either because the compounds were degraded in the roots or because their solubilities operated against translocation from root to shoot.

4. Compounds inactive *in vitro* but showing weak systemic fungicidal activity were extensively degraded in the tissues and owed their activity to an indirect mechanism, possibly by affecting the biochemistry of the host plant.

5. A systemic fungicide based on the griseofulvin structure should have high activity *in vitro*, coupled with chemical stability in the tissues and a low oil–water partition coefficient.

We are indebted to Dr J. Stubbs, Jealotts Hill Research Laboratories, for the systemic fungicidal activity tests and to Mr H. G. Hemming for the assays of activity *in vitro*.

REFERENCES

Aytoun, R. S. C. (1956). *Ann. Bot., Lond.*, **20**, 297.
Brian, P. W. (1952). *Ann. appl. Biol.* **39**, 434.
Brian, P. W., Curtis, P. J. & Hemming, H. G. (1946). *Trans. Brit. mycol. Soc.* **29**, 173.
Brian, P. W., Wright, J. M., Stubbs, J. & Way, A. M. (1951). *Nature, Lond.*, **167**, 347.

Crowdy, S. H., Gardner, D., Grove, J. F. & Pramer, D. (1955). *J. exp. Bot.* **6**, 371.
Crowdy, S. H., Green, A. P., Grove, J. F., McCloskey, P. & Morrison, A. (1959). *Biochem. J.* **72**, 230.
Crowdy, S. H., Grove, J. F., Hemming, H. G. & Robinson, K. C. (1956). *J. exp. Bot.* **7**, 42.
Davis, D. & Dimond, A. E. (1953). *Phytopathology*, **43**, 137.
Dimond, A. E. & Davis, D. (1953). *Phytopathology*, **43**, 43.
Keyworth, W. G. & Dimond, A. E. (1952). *Phytopathology*, **42**, 311.
Stokes, A. (1954). *Plant & Soil*, **5**, 132.
Stubbs, J. (1952). *Ann. appl. Biol.* **39**, 439.
van Raalte, M. H., Sijpesteijn, A. K., van der Kerk, G. J. M., Oort, A. J. P. & Pluygers, C. W. (1955). *Meded. LandbHoogesch. Gent*, **20**, 543.

The Metabolism of Glycerol in *Mycobacterium butyricum*

By N. L. EDSON, G. J. E. HUNTER, R. G. KULKA and D. E. WRIGHT
Travis Laboratory, Department of Biochemistry, Medical School, University of Otago, New Zealand

(*Received* 17 *July* 1958)

Boissevain (1943) found a trace of pyruvic acid in Long's medium on which tubercle bacilli had been grown, and suggested that the acid came from glycerol. There is evidence that glycerol is phosphorylated by the mycobacteria and converted into pyruvate according to the Embden–Meyerhof scheme (Hunter, 1953*a*; Winder & Denneny, 1955). It is known also that the mycobacteria can oxidize pyruvate to acetate (Lindsay, O'Donnell & Edson, 1950; Goldman, 1958*a*, *b*) and that the reactions of the citric acid cycle can occur in cell-free extracts of these bacteria (Faine, Whiteside & Edson, 1951; Ochoa, Stern & Schneider, 1951; Blakley, 1952; Andrejew, 1954; Yamamura, Kusunose & Kusunose, 1954, 1955; Millman & Youmans, 1954, 1955; Millman & Darter, 1956; Goldman, 1956*a*, *b*, 1957; Morrison, 1956, 1957). These facts point to a terminal oxidation of glycerol in the citric acid cycle.

Mycobacterium butyricum grows well on a phosphate-buffered synthetic medium containing glycerol as the source of carbon and ammonium ion as the source of nitrogen, and excretes into the medium a considerable amount of 2-oxoglutaric acid together with small quantities of pyruvic and succinic acid (Hunter, 1953*b*). The origin of 2-oxoglutaric acid may be ascribed to the reactions shown in Fig. 1. Guided by this hypothesis we have used ^{14}C-tracer methods to study glycerol metabolism in cultures of *M. butyricum*.

MATERIALS AND METHODS

Organism. *Mycobacterium butyricum*, no. 337, was obtained from the National Collection of Type Cultures, Lister Institute, London. For many years the stock culture has been maintained on glycerinated Dorset's egg medium in this Laboratory. It was cultivated on the synthetic medium for several generations before use in experiments. The inoculum (about 4 mg. wet wt.) was taken from a 4-day-old culture.

Medium. The synthetic medium contained 1·0 g. of $(NH_4)_2SO_4$, 0·2 g. of NaCl, 0·04 g. of $MgSO_4,7H_2O$, 10·0 g. of KH_2PO_4, 10·0 mg. of thiamine hydrochloride and approx. 30 g. of glycerol/l. of water. The pH was adjusted to 7·5 with carbonate-free 3N-NaOH solution. The required amount of $MgSO_4$ solution was added aseptically to the autoclaved medium. Glycerol and the inorganic salts were analytical reagents. Sterile solutions of radioactive substrates were added after the medium had been measured into the special culture flask used in the experiments. Solutions of radioactive glycerol and acetate were sterilized by steaming in sealed tubes for 0·5 hr. on 2 successive days; radioactive 0·5M-$NaHCO_3$ solution was sterilized by filtering it through a moist Seitz pad through which a solution of non-isotopic 0·5M-$NaHCO_3$ had been passed immediately beforehand.

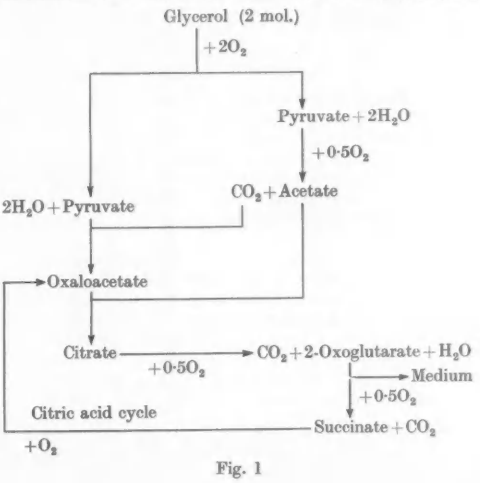


Fig. 1

Description of the apparatus. The apparatus (Fig. 2) was a closed-circuit constant-pressure macrorespirometer similar to that described by Wood, Brewer, Mickelson & Werkman (1940). It was set up in a hot room at $38 \pm 1°$.

The volume of the culture flask (*A*) was about 600 ml. and that of the medium 100 ml. The organisms grew as a surface pellicle. The ground-glass stopper of *A* carried a four-way connecting piece, one horizontal arm joining the culture flask to the gas burette (*B*) and manometer (*C*) and the parallel arm joining it to the humidifier (*D*). The short gas-inlet tube (*E*) was sealed at its lower end and provided with four holes situated about 0·5 in. from the sealed end. This arrangement prevented the down-draught from disturbing the bacterial pellicle. The upper vertical arm of the connecting piece, provided with a three-way tap and protected by a sterile cotton-wool filter (*F*), joined the closed system to a cylinder of O_2. Before introduction of O_2, the cylinder connexions and the cotton filter were flushed with the gas. The gas outlet emerged from *A* about 0·5 in. above the culture to connect with the absorbing train. The sterile zone of the flask and its immediate connexions were protected from contamination by sterile cotton-wool filters.

The 100 ml. burette (*B*), graduated to 0·2 ml., was provided with a levelling bulb (*G*), which contained a solution of NaCl saturated at 39°. The manometer was half-filled with Brodie's fluid. It was connected to a 1 l. compensating flask (*H*) to which 200 ml. of water was added to make the volume of air in *H* approximately equal to the volume of air in the closed system on the other side of the manometer.

The absorbing train consisted of (1) a glass-stoppered drying tube (*J*) containing dried $CaCl_2$ (analytical reagent) and anhydrone; (2) a CO_2-absorbing bubbler (*K*) containing 10·0 ml. of carbonate-free 3N-NaOH solution and provided with a guard tube (*L*) filled with soda asbestos and anhydrone; and (3) a guard tube (*M*) also containing soda asbestos and anhydrone. The soda asbestos and the anhydrone, both 14–22 mesh, were microanalytical reagents. *J* and *K* + *L* could be replaced as required. There were no significant changes in the weight of *M* during any of the experiments.

The air in the system was circulated at the rate of approx. 120 ml./min. by means of an all-metal bellows (*N*) and the bubbler valves (*P* and *Q*). The valves contained a saturated solution (39°) of NaCl. The bellows, which was made from four automobile petrol pumps, was driven at the

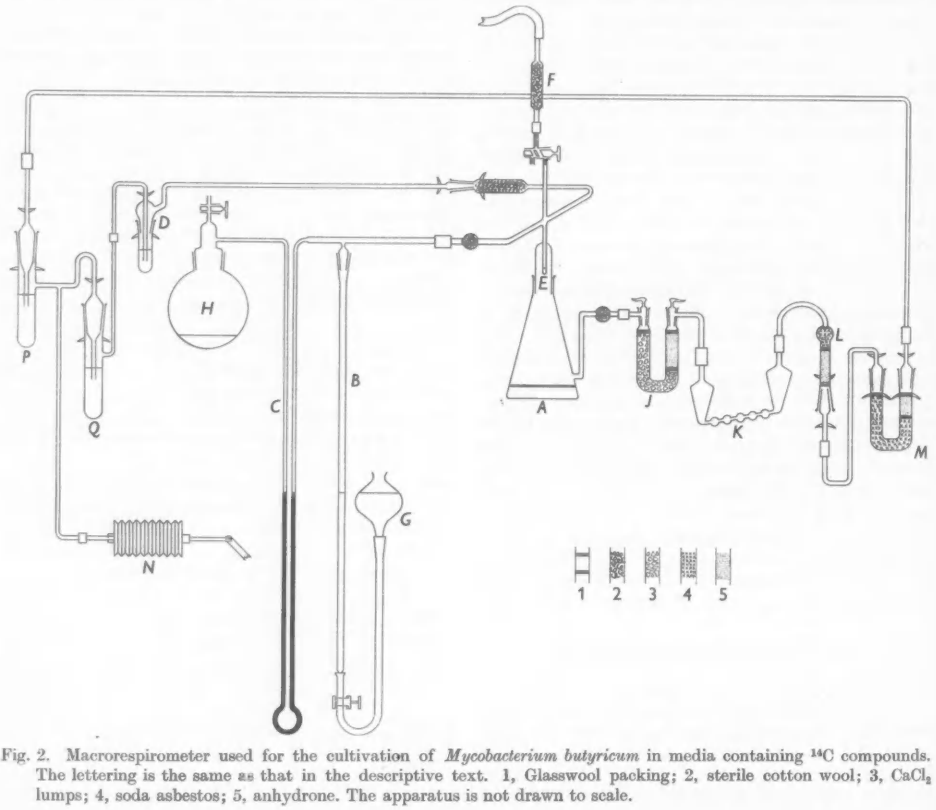


Fig. 2. Macrorespirometer used for the cultivation of *Mycobacterium butyricum* in media containing ^{14}C compounds. The lettering is the same as that in the descriptive text. 1, Glasswool packing; 2, sterile cotton wool; 3, $CaCl_2$ lumps; 4, soda asbestos; 5, anhydrone. The apparatus is not drawn to scale.

rate of 30 strokes/min. by an eccentric pulley and a geared motor.

The humidifier (*D*) contained 5 ml. of sterile water and the whole of this part of the apparatus was autoclaved before each assembly. Saturation of the air with water vapour was necessary to prevent concentration of the medium. There was no significant change in the volume of the medium in experiments lasting up to 11 days.

The ground-glass joints of the humidifier and the ground-glass surfaces within the sterile zone were lubricated with sterile rubber grease. All flexible connexions were made with thick rubber tubing.

Operation of the apparatus. The sterile medium (100 ml.) was measured into the culture flask and a solution (not more than 1 ml.) of the radioactive substrate added aseptically. After inoculation of the medium the apparatus was assembled and allowed to attain thermal equilibrium with all taps open to the air. The levelling fluid in the burette was adjusted approximately to the 100 ml. mark. After equilibration (1–2 hr.) all the taps, including those in the drying tube (*J*), were closed and the initial reading of the burette was taken with the fluid levels equalized on both sides of the manometer. All subsequent readings of the burette were taken in the same way. The barometric pressure was read at the end of the equilibration period.

The contraction of the volume (corrected to s.t.p.) of dry air in the system was the measurement of consumption of O_2. During the first 72 hr. after inoculation the uptake of O_2 was very small and circulation of the air was unnecessary. Subsequently uptake of O_2 proceeded at a considerable rate and O_2 was restored to the system intermittently when the burette reading approached zero. At each addition of O_2 the burette reading was returned to about 100 ml.

Carbon dioxide formation was determined as the increase in weight of the bubbler (*K*) and its guard tube (*L*), which were weighed as a single unit. This unit was replaced as required within the limit of its absorbing capacity, the consumption of O_2 being used as a guide to the need for replacement. The drying-tube (*J*) was replaced before the anhydrone showed any signs of moistness.

Preparation of the bacteria and the medium for analysis. At the end of an experiment the medium was decanted from the bacterial pellicle and centrifuged to remove a small quantity of bacteria. The pH was determined by a spot test with bromothymol blue. The bacterial pellicle was transferred to centrifuge tubes, broken up thoroughly and the organisms were washed three times with CO_2-free water by centrifuging at 2000 *g*. The washings were added to the medium, the total volume was measured and a 5 ml. sample reserved for the estimation of glycerol, 2-oxoglutaric and pyruvic acid.

The combined medium and washings were placed in a three-necked flask provided with (1) a gas-inlet tube, (2) a dropping-funnel and (3) a connexion to a CO_2-absorbing train similar to that described above (*J*, *K* and *L*), but of smaller capacity (3 ml. of liquid). Freshly prepared metaphosphoric acid solution (0·1 vol. of 10%, w/v, acid) was added and followed by 0·1–0·2 ml. of 10N-H_2SO_4 to bring the pH of the mixture to 3. Carbon dioxide-free air was bubbled through the flask and the bound CO_2 of the medium determined gravimetrically. The metaphosphoric acid was used to precipitate a small amount of material, designated 'protein', which the organisms release into the medium. Its composition is uncertain but it gives protein colour tests (biuret, Sakaguchi and xanthoproteic reactions). The 'protein' was removed by centrifuging, washed twice with CO_2-free water and the washings were added to the deproteinized medium.

The pH of the residual diluted medium was adjusted to 7·5 with carbonate-free 3N-NaOH solution, the volume measured and 3 ml. reserved for determination of total carbon and radioactivity. The medium was concentrated to 70–80 ml. in a rotary evaporator at 30°, acidified to pH 3 with 10N-H_2SO_4 and extracted continuously for 64 hr. with boiling peroxide-free diethyl ether. The ether was renewed at 16 and 40 hr. The ethereal extracts containing the organic acids of the medium were combined and neutralized (pH 7·5–8·0) with carbonate-free 0·5N-NaOH solution. The ether was removed *in vacuo* and the aqueous solution concentrated to 1 ml. in the rotary evaporator in preparation for chromatography.

The bacteria were dried to constant weight *in vacuo* at room temperature. A sample was reserved for combustion and measurement of radioactivity and the remainder defatted by boiling successively with 15 ml. of (*a*) ethanol–diethyl ether (1:1), (*b*) diethyl ether twice, (*c*) diethyl ether–chloroform (2:1, v/v), (*d*) ethanol–diethyl ether–conc. HCl (2:2:0·1, by vol.) and (*e*) diethyl ether. Each extraction lasted 1–2 hr. The combined extracts were shaken with water to remove HCl, dried over anhydrous Na_2SO_4 and evaporated to dryness *in vacuo*. The residual 'total lipids' were used for determination of carbon and radioactivity.

Materials. The inorganic chemicals, the acetic and succinic acid used as carriers and the benzene used as a solvent were analytical reagents. 2-Oxoglutaric acid (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) was recrystallized from ethyl acetate by addition of light petroleum. The purity of the product was at least 99% when it was assayed against the pure 2:4-dinitrophenylhydrazone of 2-oxoglutaric acid according to the method of Friedemann & Haugen (1943). [1-^{14}C]Acetate, [2-^{14}C]-acetate, [1:4-$^{14}C_2$]succinic acid, sodium [^{14}C]bicarbonate and synthetic [1:3-$^{14}C_2$]glycerol were obtained from The Radiochemical Centre, Amersham, Bucks, England.

Analytical methods

Glycerol was estimated by the method of Lambert & Neish (1950), and 2-oxoglutaric and pyruvic acid by the method of El Hawary & Thompson (1953). Carbon was determined by drying samples of organic material at 105° and performing wet combustion with the reagent of Van Slyke & Folch (1940) in the apparatus described by Sakami (1955). The CO_2 was trapped in carbonate-free 3N-NaOH solution.

Chromatographic isolation of organic acids. The stationary phase was prepared by mixing 20 g. of acid- and ether-washed Celite (no. 545, Johns Manville Corp., U.S.A.) with 20 g. of silicic acid powder (Mallinckrodt Chemical Works, New York, U.S.A.; reagent for chromatography, 100 mesh) and adding 27 ml. of 0·05N-H_2SO_4. The column was packed into a tube (2 cm. × 50 cm.) half-filled with benzene which had been equilibrated with 0·05N-H_2SO_4 and was washed with 100 ml. of benzene. The aqueous solution (1 ml.) of the salts of the organic acids extracted from the medium (see above) was acidified to pH 2–3 with 10N-H_2SO_4,

absorbed on 3 g. of the Celite–silicic acid mixture and packed on to the top of the column, which was developed with benzene and mixtures of benzene and diethyl ether containing successively greater proportions of ether. All the solvents were equilibrated with 0·05 N-H_2SO_4 and passed through filter paper before use. The fractions (10 ml.) were titrated with carbonate-free 35 mM-NaOH solution with phenol red as indicator.

This procedure, which is similar to that of Kinnory, Takeda & Greenberg (1955), was developed for the purpose of obtaining a satisfactory separation of succinic and 2-oxoglutaric acid, which did not separate satisfactorily on the Celite column of Swim & Krampitz (1954) when they were present in the proportions encountered in this work. Fig. 3 shows the degree of separation achieved from a mixture of acids (Table 1) made up in proportions which were likely to be encountered in the culture medium. In fact the cultures never contained detectable amounts of butyric and propionic acid. The recoveries are given in Table 1. There is a small overlap between pyruvic and succinic acid but 2-oxoglutaric acid is separated satisfactorily. The separation of 2-oxoglutaric and succinic acid was tested by applying a mixture of [1:4-$^{14}C_2$]succinic acid (0·1 m-mole, specific activity 14 300 counts/min./m-mole of C) and 2-oxoglutaric acid (0·83 m-mole) to the column. The succinic acid fraction contained no 2-oxoglutaric acid detectable by the method of Friedemann & Haugen (1943) and the specific activity was 14 600 counts/min./m-mole of C. Three fractions of 2-oxoglutaric acid (early, peak and late fractions) had specific activities of 23, 24 and 25 counts/min./m-mole of C. These data show that the 2-oxoglutaric acid was not significantly contaminated with succinic acid.

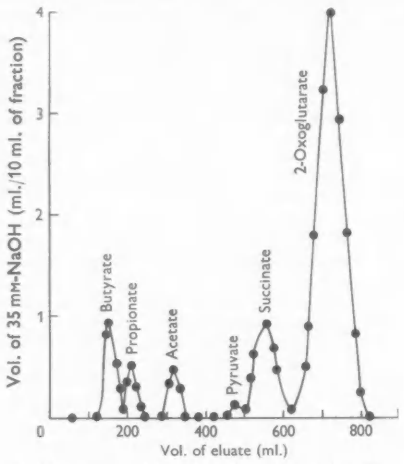


Fig. 3. Chromatographic separation of organic acids on a Celite–silicic acid column. The composition of the acid mixture is given in Table 1. Solvent: benzene, 0–130 ml.; benzene–diethyl ether (80:20, v/v) 130–190 ml., (60:40, v/v) 190–250 ml., (40:60, v/v) 250–310 ml., (30:70, v/v) 310–370 ml., (20:80, v/v) 370–430 ml., (15:85, v/v) 430–490 ml., (10:90, v/v) 490–590 ml.; diethyl ether 590–810 ml.

Table 1. *Chromatographic separation of organic acids*

Celite–silicic acid column; solvent, benzene–diethyl ether mixtures.

Acid mixture	Amount present (μmoles)	Recovery (%)
Butyric acid	93	87
Propionic acid	50	90
Acetic acid	41	107
Pyruvic acid	35	98
Succinic acid	79	
2-Oxoglutaric acid	590	91

Degradation of the organic acids separated chromatographically. The acetate fraction was acidified, distilled in steam and titrated. Carrier sodium acetate was added if necessary. Portions of the solution were evaporated to dryness and used for total combustion and for the Schmidt degradation according to Martin & Batt's (1957) modification of the method of Phares (1951).

Since pyruvic acid and succinic acid are not completely separated by the chromatographic column, the pyruvate and succinate fractions were pooled and the pyruvate decarboxylated with ceric sulphate (Krebs & Johnson, 1937). The CO_2 derived from the carboxyl group of pyruvic acid was collected in a bubbler of NaOH soln. to give a measurement of the amount of pyruvic acid present, and this value was checked by a colorimetric determination on the original pooled fractions. Ferrous sulphate was added to remove any excess of ceric sulphate and the solution extracted with diethyl ether for 24 hr. The ethereal extract was titrated, the sodium salts were taken to dryness and chromatographed on a 10 g. column of the Celite–silicic acid mixture. The acetate fraction was degraded as above to give C-2 and C-3 of the original pyruvic acid. The succinate fraction was diluted with carrier, acidified to pH 2·3, extracted with diethyl ether for 24 hr. and the ether removed. The succinic acid was purified by sublimation *in vacuo* at 115–120°. A sample was subjected to total combustion and the rest degraded according to Phares & Long (1955) except that the ethylenediamine was trapped in 0·5 N-H_2SO_4. The recovery of the methylene carbon was only 5–10%.

The 2-oxoglutarate fraction was diluted with carrier to give about 4 m-moles of the acid. A portion was degraded with ceric sulphate to give the α-carboxyl group as CO_2. The rest was totally degraded by the method of Mosbach, Phares & Carson (1951) as modified by Koeppe & Hill (1955). The specific activities of the α-carboxyl group as determined by the ceric sulphate method and by the ninhydrin reaction used in the procedure of Mosbach *et al.* (1951) agreed within 3–8%.

In general the procedures and the apparatus used were those described by Sakami (1955). The accuracy of each step was checked by calculating the specific activity of the original compound from the sum of the specific activities of its fractions and comparing this value with the specific activity found by combustion of the original compound.

Measurement of radioactivity. All samples were plated in triplicate on filter paper as $Ba^{14}CO_3$ and counted with an end-window Geiger–Müller tube. The counts were corrected for background and for self-absorption (to infinite thinness).

At least 5000 counts (standard error of less than ±1%) were recorded for each plate. The corrected counts of triplicates differed by less than 10%. The value given for each sample is the mean of the corrected counts for the three plates.

RESULTS

Utilization of glycerol and acetate

Growth of the bacteria. M. butyricum was grown on a synthetic medium (see Methods), a thin pellicle covering the surface by the end of the third day; subsequently the pellicle thickened and became deeply pigmented. In cultures 1, 2 and 3 non-isotopic glycerol was the main carbon source and smaller amounts of ^{14}C compounds were added. Culture 1 was grown in the presence of [^{14}C]-bicarbonate, culture 2 in the presence of [1-^{14}C]-acetate and culture 3 in the presence of [2-^{14}C]-acetate. In culture 4 [1:3-$^{14}C_2$]glycerol was used without the addition of another carbon source (Table 2).

Although acetate is oxidized rapidly by washed suspensions of *M. butyricum* (Hunter, 1953*b*) the organism grows very poorly when the glycerol of the medium is replaced by acetate, but it grows in the normal way when both acetate and glycerol are present. Acetate is utilized under these conditions (Tables 2–4). The experiments were designed to give a sufficient yield of 2-oxoglutaric acid with a specific activity high enough to permit the operations involved in total degradation. Table 2 shows the duration of the experiments, the yields of bacteria, the changes in the pH of the media, oxygen consumption, carbon dioxide production and the quantities of organic acids recovered from the media. The quantities of glycerol present initially and the amounts used are also given. Preliminary experiments showed that the bacteria attained their maximum growth in 7–8 days, after which slight autolysis occurred. Accumulation of 2-oxoglutaric acid, which was not significant until the fifth day after inoculation, continued for 5 days after growth had ceased. When [^{14}C]acetate was added to the medium the duration of the experiments was curtailed. It was desirable that approximately 1 m-mole of acetate should remain in the

Table 2. *Growth of* Mycobacterium butyricum *on a synthetic medium containing* ^{14}C*-labelled compounds*

The medium (100 ml.) contained $(NH_4)_2SO_4$, a salt mixture and thiamine (see Methods). In cultures 2 and 3 the carbon sources were non-isotopic glycerol and sodium [^{14}C]acetate; in culture 1 non-isotopic glycerol and [^{14}C]bicarbonate; and in culture 4 the carbon source was [^{14}C]glycerol. Initial pH of the medium, 7·5. Temp., 38±1°. Inoculum, approx. 4 mg. wet wt. of bacteria.

Culture no.	1	2	3	4
Labelled compound	[^{14}C]$NaHCO_3$	[1-^{14}C]Acetate	[2-^{14}C]Acetate	[1:3-$^{14}C_2$]Glycerol
Time (hr.)	262	168	167	283
Yield of bacteria (mg. dry wt.)	212	215	240	207
Final pH of medium	6·0	7·3	7·4	6·5
Radioactivity added (counts/min.)	4 610 000	1 770 000	1 520 000	5 550 000
Radioactivity used* (counts/min.)	504 000	1 540 000	1 490 000	1 880 000
	m-moles			
Oxygen uptake	24·1	16·5	19·4	19·9
Carbon dioxide production				
Total	17·6	13·2	15·6	15·8
Bound	0·04	1·56	2·0	0·05
Bacterial carbon	8·7	8·7	9·9	8·2
Bacterial lipid carbon	1·9	3·2	2·5	2·9
Protein carbon in the medium	0·17	0·15	0·16	0·38
Initial non-isotopic glycerol	28·6	31·7	32·0	0
Labelled compound added	0·5	7·15	6·6	32·9
Glycerol used	12·0	6·0	7·3	11·1
Acids in the medium				
2-Oxoglutaric acid	0·44	0·73	0·68	0·63
Succinic acid	0·08	0·12	0·13	0·11
Pyruvic acid	Trace	0·32	Trace	0·02
Acetic acid	0·02	1·23	0·14	0·05
	%			
Recovery of carbon†	90	97	94	90
Recovery of radioactivity‡	77	86	93	90

* In cultures 2, 3 and 4 the radioactivity used equals the sum of the counts/min. found in the CO_2, the bacteria and the medium minus the counts/min. in the residue of the added ^{14}C compound. In culture 1 the counts/min. in the CO_2 (66% of the counts/min. added) have been omitted from the sum because much of this radioactivity was expelled from [^{14}C]-bicarbonate by non-metabolic processes (see text).

† Sum of the carbon in the total CO_2, bacteria, protein of the medium and the residual medium.

‡ Sum of the counts/min. found in the same fractions of the cultures.

medium at the end of an experiment to ensure that the metabolites formed from the ^{14}C of acetate would retain a high specific activity until the termination of the experiment. This ideal was achieved in culture 2 but not in culture 3, where the added acetate had almost disappeared. Since initial concentrations of acetate exceeding 7 m-moles/100 ml. of medium diminish the yield of 2-oxoglutaric acid, it was not possible to increase the acetate concentration instead of limiting the incubation time.

Recovery of carbon and radioactivity. The carbon balance sheet was constructed by determination of the carbon in the initial medium and in the dry bacteria, the carbon dioxide (free and bound), the protein precipitated from the medium and the residual medium. The recovery of the carbon originally in the medium was 90% or more (Table 2). The recovery of added ^{14}C from cultures 2, 3 and 4 was satisfactory (86–93%) but only 77% of the added ^{14}C was recovered from culture 1.

Respiration. The rate of oxygen uptake increased sharply about 72 hr. after inoculation, reached a maximum about 120 hr. and declined slowly thereafter. The R.Q. was 0·8 in cultures 2, 3 and 4 and 0·7 in culture 1.

pH *changes and acid formation.* The medium was heavily buffered with phosphate to retard pH changes. In cultures 1 and 4 the pH decreased (cf. Hunter, 1953*b*) and there was practically no bound carbon dioxide (approx. 0·05 m-mole) in the medium at the termination of the experiments; but in cultures 2 and 3 to which acetate had been added the final and initial pH values were approximately equal in spite of acid formation. The presence of 1·56 and 2·0 m-moles of bound carbon dioxide in cultures 2 and 3 respectively shows that the pH had been stabilized by the carbonate and bicarbonate formed during the metabolism of sodium acetate (Table 2).

Bacteria. The bacteria from all the cultures were uniform in appearance and carbon content (47·4–49·5% of the dry weight). The lipid carbon was roughly one-quarter to one-third of the total bacterial carbon.

Distribution of carbon and radioactivity. The consumption of substrate carbon and its distribution amongst fractions of the cultures are shown in Table 3. About one-quarter of the carbon used is transformed into bacterial constituents and slightly less than half into carbon dioxide. The proportion of the carbon (about 10%) diverted to the formation of extracellular 2-oxoglutaric acid is relatively large. The fraction designated 'unidentified substances' in Table 3 includes free radioactive amino acids (D. E. Wright, unpublished work).

The total amount of carbon used was of the same order in all four cultures although the experiments with glycerol and acetate (cultures 2 and 3)

Table 3. *Distribution of carbon and radioactivity utilized by* Mycobacterium butyricum

These results refer to the same cultures as in Table 2.

Culture no.	Carbon source utilized (m-moles of carbon)			Distribution of carbon utilized (%)								
				Medium								
	Glycerol	Acetate	Total	CO_2	Bacteria	Protein	Succinic acid	Pyruvic acid	Acetic acid	2-Oxo-glutaric acid	Substances not recovered or unidentified
1	36·0	—	36·0	47·4	24·2	0·5	0·9	—	—	6·1	20·9
2	18·0	11·8	29·8	44·3	29·7	0·5	1·6	3·2	—	12·2	8·5
3	21·9	12·9	34·8	44·6	28·4	0·5	1·5	—	—	9·8	15·2
4	33·4	—	33·4	47·2	24·5	1·1	1·3	0·2	0·1	9·4	16·2
				Distribution of radioactivity utilized (%)								
Source of radioactivity												
Sodium [^{14}C]bicarbonate				—	62·7*	2·2*	0·14*	—	—	3·3*	31·4*
Sodium [1-^{14}C]acetate				51·6	17·8	0·2	1·0	0·1	—	6·7	22·6
Sodium [2-^{14}C]acetate				27·5	32·5	0·7	2·1	—	—	9·8	27·4
[1:3-$^{14}C_2$]Glycerol				52·8	21·2	1·0	1·2	—	0·1	7·9	15·8

* These figures are expressed as percentages of the radioactivity fixed in organic compounds only, because the radioactivity in the carbon dioxide does not have the same metabolic significance as in the other cultures.

were shorter than those with glycerol alone (cultures 1 and 4). The data suggest that acetate may be oxidized faster than glycerol.

In general the distribution of radioactivity is in parallel with that of carbon (Tables 3 and 4). One difference is conspicuous: the radioactivity derived from the methyl group of acetate is fairly evenly distributed between the bacterial carbon and the carbon dioxide (32·5 and 27·5% respectively), whereas that derived from the carboxyl group is unequally distributed between these two fractions, 51·6% appearing in carbon dioxide and 17·8% entering the bacteria. There are also differences in the specific activities of the non-lipid bacterial carbon formed from the two kinds of acetate. These distributions are consistent with the operation of a citric acid cycle that is performing synthetic functions (Weinman, Strisower & Chaikoff, 1957).

Fixation of carbon dioxide by Mycobacterium butyricum

Since the incorporation of carbon dioxide into oxaloacetate is a crucial feature of the scheme for glycerol oxidation (Fig. 1), this point was tested by adding a single dose of 0·5 m-mole of sodium [^{14}C]bicarbonate to the glycerol-containing medium before inoculation. It was expected that a substantial part of the radioactive bicarbonate would be decomposed and removed from the medium through operation of the low partial pressure of carbon dioxide in the circulating air and the development of acidity in the medium. These effects were discernible in the carbon dioxide collected from culture 1: the first sample (14·3 m-moles, 0–194 hr.) had a specific activity of 213 000 counts/min./m-mole, and the second (3·2 m-moles, 194–262 hr.) only 3150 counts/min./m-mole. In order to compensate for the anticipated removal of radioactivity by non-metabolic factors and for dilution by respiratory carbon dioxide the initial specific activity of the bicarbonate was made very high ($9{\cdot}23 \times 10^6$ counts/min./m-mole). The need for this precaution when a single dose of bicarbonate is employed is apparent in the relatively low specific activities of the acids which accumulate in the medium after 120 hr. (Table 4).

At the end of the experiment there was practically no bicarbonate left in the medium. About 11% of the radioactivity was recovered in the bacteria and the organic residues of the medium and about 66% in the respiratory carbon dioxide. Analytical errors alone will not account for the deficit. Of 0·5 m-mole of $^{14}CO_2$ added about 0·05 m-mole was fixed in organic compounds. The total yield of carbon dioxide was 17·6 m-moles, which would represent a 39-fold dilution of the remaining 0·45 m-mole of $^{14}CO_2$. The 53-fold dilution which was observed (Tables 2 and 4) could be accounted for by assuming that approximately 0·1 m-mole of $^{14}CO_2$ was lost during equilibration of the apparatus.

Tables 3 and 4 show that $^{14}CO_2$ was incorporated into the organic constituents of *M. butyricum* and into the protein and other extracellular products found in the medium. The bacterial lipid C has a much lower specific activity than the non-lipid C, whereas the protein C of the medium has a specific activity of the same order as that of the bacterial non-lipid C. Similar demonstrations of the incorporation of carbon dioxide into the substance and extracellular products of tubercle bacilli have been reported (Long, Anderson, Rittenberg, Karnovsky & Henderson, 1955; Karnovsky, Ståhlen, Suter, Long & Henderson, 1956); and Wright (1957), using the somatic proteins, has identified the amino acids which were labelled

Table 4. *Specific activities of the bacteria* (Mycobacterium butyricum) *and other products*

These results refer to the same cultures as in Table 2.

Radioactive substrate added	[^{14}C]Bicarbonate	[1-^{14}C]Acetate	[2-^{14}C]Acetate	[1:3-$^{14}C_2$]Glycerol*
	(counts/min./m-mole of C)			
Labelled atom(s) of the substrate	$9{\cdot}23 \times 10^6$	248 000	230 000	84 500
Bacterial carbon	36 400	31 500	48 000	48 500
Bacterial lipid carbon	3 800	46 300	53 500	37 700
Bacterial non-lipid carbon (calc.)	52 700	22 900	46 300	54 200
Total carbon dioxide (weighted average of successive samples)	175 000	60 300	32 600	62 600
Bound carbon dioxide	—	54 400	21 300	—
Protein of the medium	64 000	20 900	60 400	46 900
Residual acids in the medium:				
2-Oxoglutaric acid	7 600	28 200	42 800	47 000
Succinic acid	2 270	31 000	61 000	53 400
Pyruvic acid	—	7 130	—	—
Acetic acid	—	94 000	111 000	24 000

* The glycerol is assumed to be equally labelled in C-1 and C-3.

* These figures are expressed as percentages of the radioactivity fixed in organic compounds only, because the radioactivity in the carbon dioxide does not have the same metabolic significance as in the other cultures.

Table 5. *Isotope distribution in organic acids formed by* Mycobacterium butyricum *in the presence of* [^{14}C]*bicarbonate*

The medium contained non-isotopic glycerol and sodium [^{14}C]bicarbonate (initial specific activity, $9{\cdot}23 \times 10^6$ counts/min./m-mole). See culture 1, Table 2.

Metabolite		Carbon atom(s)	Specific activity (counts/min./m-mole of C)
2-Oxoglutaric acid	1	CO_2H	33 900
	2	C=O	1 040
	3	CH_2	
	4	CH_2	
	5	CO_2H	
Succinic acid	1, 4	CO_2H	4 030
	2, 3	CH_2	510

during the growth of *M. butyricum* on a synthetic medium containing glycerol and [^{14}C]bicarbonate. Aspartic and glutamic acids were heavily labelled.

According to Fig. 1 carbon dioxide is fixed in the β-carboxyl group of oxaloacetate, which subsequently enters the citric acid cycle. If citrate is metabolized asymmetrically in the manner characteristic of animal tissues (Wood, Werkman, Hemingway & Nier, 1941; Evans & Slotin, 1941; Ogston, 1948; Potter & Heidelberger, 1949), the isotope will appear predominantly in the α-carboxyl group of 2-oxoglutaric acid. Table 5 shows that the addition of [^{14}C]bicarbonate to the glycerol-containing medium leads to the formation of 2-oxoglutaric acid, in which the carbon atom of the α-carboxyl group has a specific activity about 30 times as great as the average of that found in the other carbon atoms. In this respect the result is consistent with the hypothesis. It is possible that the low concentrations of the isotope found in succinic acid and in C-2 to C-5 of oxoglutaric acid were introduced by means of pyruvate, which had been labelled in all three carbon atoms by the following process: $^{14}CO_2$ can be introduced into the carboxyl group of pyruvate by way of a symmetrical dicarboxylic acid, e.g. fumaric or succinic. Incorporation of carboxyl-labelled pyruvate into hexose, followed by metabolism of the hexose in the pentose phosphate cycle, which occurs in the mycobacteria (Crowley, 1955), would yield pyruvate labelled in all its carbon atoms.

Succinic and 2-oxoglutaric acid are not in isotopic equilibrium with each other.

Metabolism of acetate

Another essential part of the hypothesis (Fig. 1) is the postulate that acetate derived from glycerol is oxidized in the citric acid cycle. The oxidation of acetate was studied by adding carboxyl- or methyl-labelled acetate to the glycerol-containing medium and determining the isotope distribution in 2-oxoglutaric acid and other acids recovered from the medium (Table 6). It will be seen that the residual extracellular acetate has acquired a low concentration of ^{14}C in the carbon atom which was originally unlabelled and that the specific activity of the carbon atom bearing the original label has diminished. This effect shows that the external acetate has exchanged with intracellular acetate, but the specific activities indicate that the external acetate has not attained isotopic equilibrium with the internal acetate pool.

The isotope distributions in the 2-oxoglutaric and succinic acid formed in the presence of [1-^{14}C]- and [2-^{14}C]-acetate conform to the well-known patterns of labelling which can be deduced from the theory of the citric acid cycle by application of principles enunciated by Strisower, Kohler & Chaikoff (1952) and Weinman *et al.* (1957). These principles assume that acetate of constant specific activity enters the cycle at a constant rate. Such conditions do not hold for cultures of *M. butyricum*. The mass of bacteria increases to reach a maximum about the eighth day and the concentration of external acetate diminishes progressively, most of this acetate vanishing by the end of the experiment. The excess of glycerol, however, is so great that the final concentration in the medium is about four-fifths of the initial concentration (Table 2). In these circumstances there will be a progressive decline in the specific activity of the internal acetate pool owing to dilution with non-isotopic acetate formed from glycerol. An indication of the correctness of this supposition is to be found in the specific activity of the carbon dioxide, the later samples having a significantly lower isotope concentration (Table 6).

If acetate labelled in one carbon atom only enters the citric acid cycle, the distribution of the isotope in the intermediates of the cycle and in carbon dioxide can be calculated from the equations of Strisower *et al.* (1952). After an infinite number of cycles the distribution would approach the limits given in Table 7 if there were no dilution by unlabelled oxaloacetate. Although dilution does occur, Table 7 is a guide to the expected ^{14}C ratios between the carbon atoms.

Table 6. *Isotope distribution in the carbon dioxide and organic acids formed by* Mycobacterium butyricum *in the presence of* [^{14}C]*acetate*

The medium contained non-isotopic glycerol and either [1-^{14}C]acetate (248 000 counts/min./m-mole of C-1) or [2-^{14}C]-acetate (230 000 counts/min./m-mole of C-2). See cultures 2 and 3, Table 2.

			Specific activity (counts/min./m-mole of C)	
Labelled substrate added			[1-^{14}C]Acetate	[2-^{14}C]Acetate
Metabolite	Carbon atom(s)			
2-Oxoglutaric acid	1	CO_2H	57 500	21 000
	2	C=O	530	50 800
	3	CH_2	980	49 200
	4	CH_2	370	62 000
	5	CO_2H	84 400	12 900
Succinic acid	1, 4	CO_2H	58 800	19 600
	2, 3	CH_2	880	46 400
Pyruvic acid	1	CO_2H	20 100	—
	2	C=O	1 060	—
	3	CH_3	235	—
Acetic acid (residual)	1	CO_2H	184 000	8 960
	2	CH_3	1 730	205 000
CO_2	—	—	65 600 (0–129 hr.) 56 100 (129–168 hr.)	46 800 (0–126 hr.) 21 300 (126–167 hr.)
Bound CO_2	—	—	54 400	21 300

Table 7. *Theoretical distribution of acetate carbon entering the citric acid cycle or the glyoxylate cycle*

The specific activity of the labelled carbon atom of acetate is taken to be 1·0. It is assumed that there is no dilution either by non-isotopic acetate or by non-isotopic oxaloacetate and that isotope equilibrium has been reached.

	Limit of specific activity			
	[1-^{14}C]Acetate		[2-^{14}C]Acetate	
Cycle	Citric acid	Glyoxylate	Citric acid	Glyoxylate
Oxaloacetate				
CO_2H	0·5	1·0	0·5	0
C=O	0	0	1·0	1·0
CH_2	0	0	1·0	1·0
CO_2H	0·5	1·0	0·5	0
2-Oxoglutarate*				
CO_2H	0·5	1·0	0·5	0
C=O	0	0	1·0	1·0
CH_2	0	0	1·0	1·0
CH_2	0	0	1·0	1·0
CO_2H	1·0	1·0	0	0
Succinate				
CO_2H	0·5	1·0	0·5	0
CH_2	0	0	1·0	1·0
CH_2	0	0	1·0	1·0
CO_2H	0·5	1·0	0·5	0
CO_2 from cycle	0·5	—	0·5	—
Pyruvate				
CO_2H	0·5	1·0	0·5	0
C=O	0	0	1·0	1·0
CH_3	0	0	1·0	1·0

* 2-Oxoglutaric acid is not a catalytic component of the glyoxylate cycle but is taken to be a product of it.

The specific activities of C-4 and C-5 of 2-oxoglutaric acid (Table 6) may be regarded as the mean specific activities of the methyl and carboxyl groups of the intracellular acetate during the period over which oxoglutaric acid was excreted into the medium, i.e. from about 120 hr. until the end of the experiments. This assumption about the status of the intracellular acetate pool is consistent with the observed distribution of ^{14}C in both samples of 2-oxoglutaric acid. The virtual exhaustion of the added [2-^{14}C]acetate in culture 3 (Table 2) will account for the fact that the ratio of the specific activities of C-4 and C-5 in 2-oxoglutaric acid is only 5:1; whereas in culture 2, in which the residual [1-^{14}C]acetate was 1·23 m-moles, the ratio of the specific activities of C-5 and C-4 is 240:1.

It will be noticed that the α-carboxyl group of 2-oxoglutaric acid is in isotopic equilibrium with the later sample of carbon dioxide (Table 6). This is to be expected because most of the oxoglutaric acid is formed after 120 hr. The succinic acid is in isotopic equilibrium with the moiety of oxoglutaric acid (C-1, C-2 and C-3) which is derived from oxaloacetate. Culture 2 alone gave a sufficient yield of pyruvic acid to permit degradation. All the carbon atoms were labelled but the pyruvic acid is not in isotopic equilibrium with the succinic acid.

Neither sample of 2-oxoglutaric acid has reached a theoretical equilibrium like that discussed by Strisower *et al.* (1952) or shown in Table 7. The specific activity of the α-carboxyl group in the sample derived from [1-^{14}C]acetate is significantly greater than the predicted value (about 42 000 counts/min./m-mole of C); and in the sample derived from [2-^{14}C]acetate it is significantly lower than the predicted 37 000 counts/min./m-mole of C. Moreover, the equal specific activities of C-2 and C-3 in the sample derived from [2-^{14}C]acetate are significantly lower than the specific activity of C-4. These facts may reflect the complex kinetics of the cultures in which the rates of growth, substrate disappearance and acid liberation are important variables. They may also imply the operation of a metabolic process additional to the classical steps of the citric acid cycle.

Metabolism of glycerol

Tables 4 and 8 give the specific activities of the products formed by *M. butyricum* in a medium containing [1:3-$^{14}C_2$]glycerol. Since the substrate is deemed to be equally labelled in C-1 and C-3, the subsequent distribution of the isotope will not be influenced by asymmetric metabolism of glycerol (Schambye, Wood & Popják, 1954; Swick & Nakao, 1954) if this process occurs in *M. butyricum*. According to hypothesis (Figs. 1, 4), the acetate formed from glycerol should be labelled more heavily in the methyl than the carboxyl group; carbon dioxide released in the oxidative decarboxylation of pyruvate should be labelled; and carbon dioxide fixation should give rise to oxaloacetate labelled in both carboxyl groups and more heavily in the methylene than in the carbonyl group. Since there was a considerable excess of labelled glycerol in the medium at the termination of the experiment, one of the complications of previous experiments was avoided, the only ^{12}C available to dilute the isotope being C-2 of glycerol.

It follows that the isotope distribution in 2-oxoglutaric acid should resemble the pattern obtained when [2-^{14}C]acetate is metabolized but

Table 8. *Isotope distribution in the carbon dioxide and organic acids formed by* Mycobacterium butyricum *in the presence of* [^{14}C]*glycerol*

The medium contained [1:3-$^{14}C_2$]glycerol (specific activity, 56 300 counts/min./m-mole of C or 84 500 counts/min./m-mole of C in each labelled atom). See culture 4, Table 2.

Metabolite	Carbon atom(s)		Specific activity (counts/min./m-mole of C)
2-Oxoglutaric acid	1	CO_2H	39 600
	2	C=O	66 500
	3	CH_2	47 000
	4	CH_2	73 400
	5	CO_2H	9 380
Succinic acid	1, 4	CO_2H	40 600
	2, 3	CH_2	60 200
Acetic acid	1	CO_2H	8 100
	2	CH_3	48 600
CO_2	—	—	73 700 (0–126 hr.)
			59 300 (126–190 hr.)
			53 600 (190–283 hr.)

should be modified by an influx of labelled oxaloacetate into the cycle. The results are consistent with this argument (Table 8). Again, the labelling in C-4 and C-5 may be regarded as indicative of the average labelling of the methyl and carboxyl groups of acetate in the intracellular pool. The small amount of acetate recovered from the medium is not likely to be in isotopic equilibrium with the internal pool (Swim & Krampitz, 1954). If [2-^{14}C]-acetate were the only labelled substrate entering the cycle, C-2, C-3 and C-4 of 2-oxoglutaric acid should be equally labelled when isotopic equilibrium is attained (Table 7). In fact the specific activity of C-3 is significantly lower than that of C-2 and C-4. This can be explained by influx of oxaloacetate, the non-randomized labelling of which would maintain the concentration of isotope in C-2, but dilute that in C-3. The succinic acid is in isotopic equilibrium with the 2-oxoglutaric acid.

The earliest sample of carbon dioxide collected during the phase of rapid growth has a high specific activity which can be explained by assuming that a significant fraction of this carbon dioxide has arisen from the oxidative decarboxylation of pyruvate, whilst an equivalent amount of the resulting acetate has been used for synthetic purposes. The later samples of carbon dioxide have specific activities consistent with complete combustion of glycerol. Neither the carboxyl group of succinic acid nor the α-carboxyl group of 2-oxoglutaric acid is in isotopic equilibrium with the carbon dioxide; yet both these carboxyl groups possess isotope concentrations which would be predicted from the observed specific activities in C-4 and C-5 of 2-oxoglutaric acid (see Fig. 4).

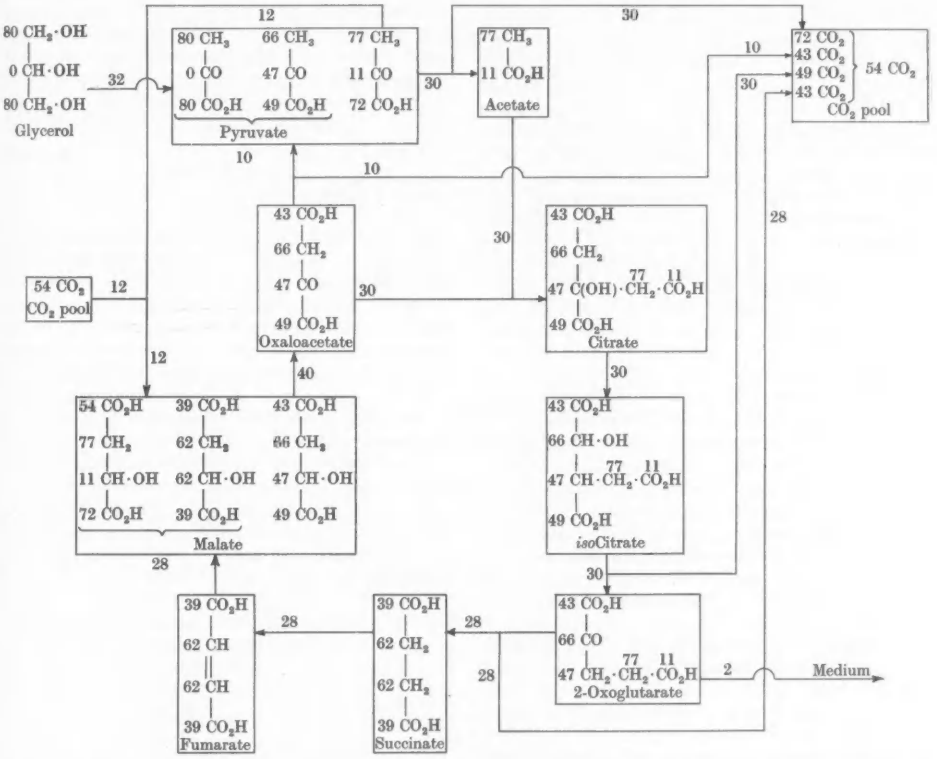


Fig. 4. A model to illustrate a possible steady-state distribution of ^{14}C resulting from the metabolism of [1:3-$^{14}C_2$]glycerol. The numbers adjacent to the carbon atoms represent specific activities $\times 10^{-3}$; those adjacent to the arrows represent molar quantities of metabolites transferred between the metabolic pools.

DISCUSSION

It has been established that $^{14}CO_2$ is fixed into 2-oxoglutaric acid in the position to be expected if a citric acid cycle operates in *M. butyricum* (Table 5). Further, the metabolism of [1-^{14}C]acetate and [2-^{14}C]acetate has led to the production of 2-oxoglutaric and succinic acid in which the isotope distribution (Table 6) is consistent with the view that these substances are formed by way of the citric acid cycle.

The results obtained with [1:3-$^{14}C_2$]glycerol permit amplification of the original hypothesis outlined in Fig. 1. The model shown in Fig. 4 depicts a steady-state mechanism by means of which 2-oxoglutarate and succinate with an isotope distribution similar to that observed could be formed from [1:3-$^{14}C_2$]glycerol. Whole numbers adjacent to the carbon atoms represent the approximate specific activities ($\times 10^{-3}$). The specific activity of each labelled carbon atom in [1:3-$^{14}C_2$]-glycerol is taken to be 80 in order that the resultant specific activities may be comparable with those observed (Table 8). The numbers beside the arrows denote molar quantities of the molecular species mixed in the metabolic pools. These values have been chosen arbitrarily to fit the observed isotope distribution. The outflow of carbon from the citric acid cycle for the synthesis of carbohydrates, amino acids and lipids has been neglected and only the production of 2-oxoglutaric acid has been considered.

Essentially the scheme shows the distribution of ^{14}C from a species of acetate which is labelled predominantly in the methyl group. The acetate is in isotopic equilibrium with the pyruvate pool, one-quarter of which has arisen from oxaloacetate and three-quarters directly from glycerol. Since the mechanism of carbon dioxide fixation is not known, it has been assumed that fixation occurs by way of malate, which mixes with cycle malate in the ratio of 12:28, and that oxaloacetate arises solely from the malate pool. The possibility of randomization in a malate–fumarate equilibrium has been omitted. The resulting isotope distribution in 2-oxoglutaric acid is very similar to that found by experiment, the distinctive feature being the unequal distribution of ^{14}C between C-2 and C-3 (cf. Tables 6 and 8).

In Table 6 the specific activity of C-1 of the 2-oxoglutaric formed in the presence of [1-^{14}C]acetate is higher than would be predicted from the citric acid cycle equilibrium (Table 7); and in the 2-oxoglutaric acid formed in the presence of [2-^{14}C]-acetate the corresponding specific activity is lower than would be predicted. Such effects might be expected if the recently described glyoxylate cycle (Kornberg & Madsen, 1957; Kornberg & Krebs, 1957; Kornberg & Quayle, 1958) were operating in *M. butyricum* (see also Table 7). Although *iso*-citratase has been found in this organism (Morrison & Wright, 1958), the occurrence of a glyoxylate cycle is uncertain because the presence of malate synthetase has not been demonstrated.

The scheme of Fig. 4 illustrates an attempt to find one way in which the observed distribution could have arisen, but the complicated dynamics of growing cultures make it difficult to interpret metabolic events from the observation of final isotope distributions. Despite this obstacle, a key to interpretation has been found in the isotopic composition of an unusually large excretion of 2-oxoglutaric acid, the distinctive labelling pattern of which strongly supports the view that the citric acid cycle plays a major part in the intermediary metabolism of glycerol.

SUMMARY

1. The metabolism of glycerol has been studied quantitatively in cultures of *Mycobacterium butyricum* grown on a synthetic medium containing [^{14}C]glycerol or non-isotopic glycerol accompanied by [^{14}C]bicarbonate or [^{14}C]acetate. The organism excreted a considerable amount of 2-oxoglutaric acid into the medium.

2. The incorporation of ^{14}C into the bacteria and some of their extracellular products has been determined.

3. Total degradation of the 2-oxoglutaric acid formed in the presence of sodium [^{14}C]bicarbonate gave a labelling pattern consistent with the fixation of carbon dioxide in the β-carboxyl group of precursor oxaloacetate; and the patterns found in the presence of [1-^{14}C]- or [2-^{14}C]-acetate are consistent with oxidation of acetate in the citric acid cycle.

4. The distribution of ^{14}C in the 2-oxoglutaric acid formed in the presence of [1:3-$^{14}C_2$]glycerol is consistent with the scheme: 2 glycerol $\rightarrow$ 2 pyruvate $\rightarrow$ oxaloacetate + acetate $\rightarrow$ 2-oxoglutarate + carbon dioxide. The scheme is discussed in detail.

We wish to thank the W. H. Travis Trustees of Christchurch, New Zealand, for their generous support of this work and Professor Harland G. Wood (Fulbright Professor visiting this Laboratory in 1955) for his invaluable advice on the use of isotopic-tracer techniques.

REFERENCES

Andrejew, A. (1954). D.Sc. Thesis: Faculty of Sciences, University of Paris.

Blakley, R. L. (1952). *J. Bact.* **64**, 609.

Boissevain, C. H. (1943). *Proc. Soc. exp. Biol., N.Y.*, **54**, 342.

Crowley, G. M. (1955). *Proc. Univ. Otago med. Sch.* **33**, 22.

El Hawary, M. F. S. & Thompson, R. H. S. (1953). *Biochem. J.* **53**, 340.

Evans, E. A. & Slotin, L. (1941). *J. biol. Chem.* **141**, 439.
Faine, S., Whiteside, M. & Edson, N. L. (1951). *Proc. Univ. Otago med. Sch.* **29**, 18.
Friedemann, T. E. & Haugen, G. E. (1943). *J. biol. Chem.* **147**, 415.
Goldman, D. S. (1956*a*). *J. Bact.* **71**, 733.
Goldman, D. S. (1956*b*). *J. Bact.* **72**, 401.
Goldman, D. S. (1957). *J. Bact.* **73**, 602.
Goldman, D. S. (1958*a*). *Biochim. biophys. Acta*, **27**, 506.
Goldman, D. S. (1958*b*). *Biochim. biophys. Acta*, **27**, 513.
Hunter, G. J. E. (1953*a*). *Biochem. J.* **55**, 320.
Hunter, G. J. E. (1953*b*). *J. Bact.* **66**, 676.
Karnovsky, M. L., Stählen, H., Suter, E., Long, E. R. & Henderson, H. J. (1956). *Proceedings of the International Conference on the Peaceful Uses of Atomic Energy*, vol. 10, p. 509. New York: United Nations.
Kinnory, D. S., Takeda, Y. & Greenberg, D. M. (1955). *J. biol. Chem.* **212**, 379.
Koeppe, R. E. & Hill, R. J. (1955). *J. biol. Chem.* **216**, 813.
Kornberg, H. L. & Krebs, H. A. (1957). *Nature, Lond.*, **179**, 988.
Kornberg, H. L. & Madsen, N. B. (1957). *Biochim. biophys. Acta*, **24**, 651.
Kornberg, H. L. & Quayle, J. R. (1958). *Biochem. J.* **68**, 542.
Krebs, H. A. & Johnson, W. A. (1937). *Enzymologia*, **4**, 148.
Lambert, M. & Neish, A .C. (1950). *Canad. J. Res.* B, **28**, 83.
Lindsay, M., O'Donnell, T. V. & Edson, N. L. (1950). *Biochem. J.* **46**, 248.
Long, E. R., Anderson, R. J., Rittenberg, D., Karnovsky, M. L. & Henderson, H. J. (1955). *Amer. Rev. Tuberc.* **71**, 609.
Martin, J. K. & Batt, R. D. (1957). *J. Bact.* **74**, 359.
Millman, I. & Darter, R. W. (1956). *Proc. Soc. exp. Biol. N.Y.*, **91**, 271.
Millman, I. & Youmans, G. P. (1954). *J. Bact.* **68**, 411.
Millman, I. & Youmans, G. P. (1955). *J. Bact.* **69**, 320.
Morrison, N. E. (1956). *Proc. Univ. Otago med. Sch.* **34**, 23.
Morrison, N. E. (1957). *Proc. Univ. Otago med. Sch.* **35**, 35.
Morrison, N. E. & Wright, D. E. (1958). *Proc. Univ. Otago med. Sch.* **36**, 15.
Mosbach, E. H., Phares, E. F. & Carson, S. F. (1951). *Arch. Biochem. Biophys.* **33**, 179.
Ochoa, S., Stern, J. R. & Schneider, M. C. (1951). *J. biol. Chem.* **193**, 691.
Ogston, A. G. (1948). *Nature, Lond.*, **162**, 963.
Phares, E. F. (1951). *Arch. Biochem. Biophys.* **33**, 173.
Phares, E. F. & Long, M. V. (1955). *J. Amer. chem. Soc.* **77**, 2556.
Potter, V. R. & Heidelberger, C. (1949). *Nature, Lond.*, **164**, 180.
Sakami, W. (1955). *Handbook of Isotope Tracer Methods.* Ed. by Sakami, W., Dept. Biochem., Western Reserve University School of Medicine, Cleveland, Ohio, U.S.A.
Schambye, P., Wood, H. G. & Popják, G. (1954). *J. biol. Chem.* **206**, 875.
Strisower, E. H., Kohler, G. D. & Chaikoff, I. L. (1952). *J. biol. Chem.* **198**, 115.
Swick, R. W. & Nakao, A. (1954). *J. biol. Chem.* **206**, 883.
Swim, H. E. & Krampitz, L. O. (1954). *J. Bact.* **67**, 419.
Van Slyke, D. D. & Folch, J. (1940). *J. biol. Chem.* **136**, 509.
Weinman, E. O., Strisower, E. H. & Chaikoff, I. L. (1957). *Physiol. Rev.* **37**, 252.
Winder, F. G. & Denneny, J. M. (1955). *Nature, Lond.*, **175**, 636.
Wood, H. G., Brewer, C. R., Mickelson, M. N. & Werkman, C. H. (1940). *Enzymologia*, **8**, 314.
Wood, H. G., Werkman, C. H., Hemingway, A. & Nier, A. O. (1941). *J. biol. Chem.* **139**, 483.
Wright, D. E. (1957). *Proc. Univ. Otago med. Sch.* **35**, 37.
Yamamura, Y., Kusunose, M. & Kusunose, E. (1954). *J. Biochem., Tokyo*, **39**, 513.
Yamamura, Y., Kusunose, M. & Kusunose, E. (1955). *J. Biochem., Tokyo*, **42**, 785.

Enzymic Studies of X-Irradiated Cornea and Lens with Special Reference to Glutathione Reductase

By R. E. KUHLMAN and R. A. RESNIK

Ophthalmology Branch, National Institute of Neurological Diseases and Blindness, National Institutes of Health, Public Health Service, U.S. Department of Health, Education and Welfare, Bethesda, Maryland

(*Received* 21 *April* 1958)

Nordmann & Mandel (1955) and Mandel & Schmitt (1956) have reported a decrease in lens hexokinase activity to be an early change after X-irradiation of the eye. van Heyningen & Pirie (1957) have not been able to confirm these results. The question of whether hexokinase activity is affected in the first few days after exposure to irradiation is important because hexokinase has been shown by Green, Bocher & Leopold (1955) to be a limiting system in the glycolytic pathway of lens tissue. The relatively low levels found in cornea suggest that it may be limiting there also (Kuhlman & Resnik, 1958; Kuhlman, 1959). A decrease in the activity of this or any other system related directly to metabolism might be expected to produce a morphological change in the tissue. Attempts to correlate lens damage induced by radiation with early enzymic changes have not been successful (Pirie, van Heyningen & Boag, 1953; van Heyningen, Pirie & Boag, 1954). With the exception of an early fall of

lens glutathione content, such biochemical changes as have been described were noted at a time far removed from initial exposure to irradiation and may represent complicating factors in a severely damaged tissue (Pirie, van Heyningen & Flanders, 1955). Similar studies have not been reported for the cornea.

The present study was prompted, in part, by the discrepancy of findings concerning the effect of X-irradiation on lens hexokinase activity and by the morphological observations of the effect of irradiation on cornea (Friedenwald & Sigelman, 1953) and lens (von Sallmann, 1957). Since relatively low doses of ionizing radiation have been shown by von Sallmann *et al.* (1955) to produce early cytological changes in the lens epithelium, it is possible that a biochemical lesion might precede or accompany them. Corneal epithelium was used in part of this work because of its high mitotic activity (Friedenwald, Buschke & Moses, 1948), which has been shown by Friedenwald & Sigelman (1953) to be affected both in rate and morphology by doses of X-irradiation from 4 to 1000 r. Corneal epithelium lends itself especially well to handling by the micro-techniques used. Enzymically, it is much more active than lens or lens epithelium and capsule.

This study deals with the enzymic activity of whole lens, cornea and corneal epithelium 1, 4 and 14 days after exposure to irradiation. Hexokinase was examined for the reasons already mentioned. Glutathione reductase was studied since Pirie *et al.* (1953) found that its activity decreased in the lens after X-irradiation, after a 3- to 5-month latency period. *iso*Citric dehydrogenase was investigated in the corneal epithelium as a representative member of the citric acid cycle. The biological effects of the irradiation were observed with the biomicroscope and counts were made of the dividing cells present in the lens epithelium from one animal of each group.

METHODS AND MATERIAL

Twelve white rabbits, about 3 months old, lightly anaesthetized with Nembutal and provided with total body shielding but for the right eye, were given 1000 r. at the rate of 100 r./min. from a 210 kv X-ray source filtered with 0·5 mm. Cu and 1 mm. Al. The beam was directed at the right eye in such a manner as to avoid the opposite eye, which served as a control. Six animals were killed at 4 and 14 days after irradiation. The eyes were enucleated and frozen in liquid nitrogen. Frozen sections of the cornea were cut and lyophilized at $-20°$ as described by Lowry (1953) and Lowry, Roberts, Leiner, Wu & Farr (1954). Samples of epithelium (0·5–1·5 μg.) were obtained and treated as described previously.

In a second series, 12 white rabbits, 10–12 weeks old, under light Nembutal anaesthesia, were given 1000 r., a lead cone being used to direct the beam to the right eye. The left eye served as a control. The other radiation factors were changed by reducing the thickness of the Cu and Al filters to half those used in the first series. At intervals of 1, 4 and 14 days after irradiation, the lenses and corneas were removed and homogenized in cold 0·025 M-sodium phosphate buffer, pH 7·4. (All pH values were measured with a model G Beckman pH meter.) The corneas were crushed and the tough insoluble stromal material was removed. Hexokinase assays were performed immediately.

The animals were observed with the biomicroscope before and after irradiation. Counts of mitotic figures were made on whole-mount preparations of lens epithelium from one animal of each group by the procedure of von Sallmann, Caravaggio, Munoz & Drungis (1957).

Hexokinase and *iso*citric dehydrogenase were assayed by the methods of O. H. Lowry (personal communication), Buell, Lowry, Roberts, Chang & Kapphahn (1958) and Robins (1957). Because the activity of hexokinase in a lens homogenate is low, it was necessary to reduce the blank as much as possible by using strong alkali (Lowry, Roberts & Kapphahn, 1957) to measure fluorescence of reduced triphosphopyridine nucleotide (TPNH).

Assay of glutathione reductase. Methods used to assay glutathione reductase in lens have been complicated by the inhibition caused by the high concentration of free glutathione (up to 10 mM) in this tissue. van Heyningen & Pirie (1953) dialysed the lens homogenate and assayed the enzyme by coupling it with another system to produce TPNH. As these procedures are not suitable for micro-analysis a sensitive method requiring no predialysis was developed. The inhibitory levels of reduced glutathione (GSH) were lowered by diluting the tissue 500–1000 times.

The pH for optimum activity of the enzyme is 7·2 (van Heyningen & Pirie, 1953). Samples of corneal epithelium (1–2 μg.) were placed in micro-test tubes and covered with 10 μl. of ice-cold medium consisting of 0·1 M-sodium phosphate buffer, pH 7·2, bovine serum albumin (0·05%), ethylenediamine tetra-acetic acid (disodium salt; 0·5 mM), nicotinamide (25 mM), oxidized glutathione (1·5 mM) and TPNH (0·4 mM). The pH of the final reaction mixture was measured on the model G Beckman pH meter. The samples were incubated at 38° for 1 hr. The tubes were then removed and placed in an ice bath, and 3 μl. of 2 M-HCl was added to stop the reaction. The tubes were warmed for 3–4 min. at 38° to destroy any remaining TPNH. Portions of 10 μl. were then added to 50 μl. of 10 M-NaOH in 3 ml. fluorimeter tubes and the contents thoroughly mixed. This converts the oxidized triphosphopyridine nucleotide (TPN^+) formed in the reaction into the form which can be measured fluorimetrically (Lowry *et al.* 1957). After 1 hr. at room temperature the samples were diluted with 1 ml. of water and read in a Farrand fluorimeter with primary filter Corning no. 5860 and secondary filters nos. 3387, 5562 and 4308. Blanks and standards were obtained by adding 1 μl. of water and 1 μl. of a standard containing 3 μm-moles of TPN^+ to the incubation medium containing no sample.

Lens homogenate was also assayed with the method by using a 2 μl. sample containing 50–200 μg. wet wt. of lens in place of the sample of corneal epithelium. Since the concentration of GSH of lens exceeds that of cornea, the method was tested with lens to assure a linear relation of activity with respect to incubation time and to assure freedom from GSH inhibition.

Table 1. *Hexokinase and glutathione reductase in homogenates of X-irradiated lens and cornea*

Activity is expressed as m-moles of substrate converted/kg. of wet tissue/hr. Values are means ± S.E.M. from the number of animals indicated in parentheses.

Time after irradiation	Hexokinase		Glutathione reductase	
	Lens	Cornea	Lens	Cornea
24 hr.	6·42 ± 0·62 (4)	25·5 ± 2·5 (3)	10·4 ± 0·59 (4)	40·1 ± 7·0 (3)
4 days	5·67 ± 0·45 (4)	26·7 ± 3·0 (4)	8·95 ± 0·68 (4)	45·1 ± 5·9 (4)
14 days	4·83 ± 0·27 (4)	29·7 ± 2·5 (4)	8·77 ± 0·48 (4)	30·4 ± 2·9 (4)
Control	5·73 ± 0·55 (9)	30·0 ± 6·5 (8)	9·36 ± 0·71 (9)	38·4 ± 4·8 (8)

Table 2. *Enzyme activity in control and irradiated corneal epithelium*

Activity is expressed as moles of substrate converted/kg. of dry tissue/hr. Values are means ± S.E.M. from the number of animals indicated in parentheses. Four to five determinations were done on each animal.

Time after irradiation	Hexokinase	Glutathione reductase	*iso*Citric dehydrogenase
Control	0·825 ± 0·059 (4)	1·50 ± 0·13 (6)	8·36 ± 1·3 (4)
4 days	0·955 ± 0·17 (6)	1·54 ± 0·14 (6)	9·76 ± 0·62 (5)
14 days	0·932 ± 0·091 (4)	1·52 ± 0·22 (5)	9·24 ± 0·86 (5)

RESULTS AND DISCUSSION

As in the results of previous reports on biomicroscopic examination of eyes which have been exposed to 1000 r., evidence of damage was limited to the appearance of a number of cells in the aqueous humour. Cells were also observed over the lens surface in the pupillary area. By the fourth day these changes disappeared. The cornea and lens appeared normal. Counts of the cells in mitosis in flat preparations of the lens epithelium were consistent with the findings of von Sallmann *et al.* (1957).

Hexokinase. There was no significant decrease in hexokinase activity in homogenates of lens or cornea 1, 4 or 14 days after irradiation (Table 1). The activity of hexokinase in lens obtained by this method is in good agreement with that observed by van Heyningen & Pirie (1957). Hexokinase in the corneal epithelium was likewise unaffected by the irradiation (Table 2). These results confirm the findings of van Heyningen & Pirie (1957). It appears that hexokinase is not as labile as Nordmann & Mandel (1955) and Mandel & Schmitt (1956) reported.

Glutathione reductase. To determine whether the amount of GSH in lens tissue, and that formed by the reduction of oxidized glutathione is sufficient to inhibit the reaction, a threefold range of lens samples were assayed for activity (Fig. 1). The activity was not significantly diminished with lens samples up to 200 μg. wet wt. (40 μg. dry wt.). The decrease in activity for samples above 200 μg. wet wt. suggests that the substrate in the medium has become a limiting factor. On the basis of 320 mg. of GSH/100 g. of rabbit lens wet wt. (Pirie & van Heyningen, 1956), a 200 μg. sample would contain

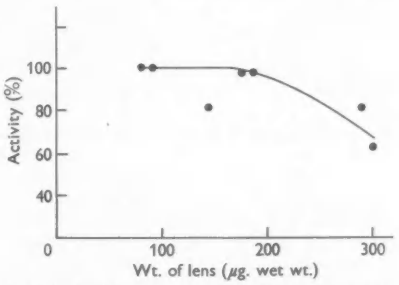


Fig. 1. Relationship between glutathione reductase activity and the size of lens sample. The procedure for the assay of glutathione reductase described in the text was followed. A portion (2–3 μl.) of lens homogenate, containing the wet wt. of lens indicated on the abscissa, was added to 10 μl. of the substrate mixture in each case.

2·08 μm-moles of GSH, and the final GSH concentration in the 12 μl. incubation volume would be 0·17 mM. Fig. 2 shows the effect of added GSH on the activity of lens homogenates. Significant inhibition of the enzyme occurs only at GSH concentrations in excess of 0·5 mM. The linear relationship between increase in fluorescence and incubation time up to 1 hr. (Fig. 3) indicates that the enzyme is stable under the conditions used for assay. To determine if the enzyme would withstand lyophilization, the activity of fresh brain and liver homogenate was compared with lyophilized preparations of these tissues and found to be equivalent, after accounting for water content. Lyophilized tissue stored *in vacuo* at −30° for 2 months did not lose any activity. Similar observations of the stability of glutathione reductase have been reported by Pihl, Eldjarn & Bremer (1957).

In order to compare the results obtained in this work with those reported by van Heyningen & Pirie (1953), their values reported as change in extinction/10 min./g. (wet wt.) of lens tissue were converted into m-moles of TPN^+/kg. wet wt./hr. The values of 0·72–3·74 m-moles of TPN/kg. wet wt./hr. thus calculated are lower than those obtained in this work. The lower values may be the result of either loss of activity during dialysis or failure of the lens malic enzyme system to maintain optimum conditions. Table 3 shows a comparison of glutathione reductase activity in several locations. Corneal epithelium has roughly about 50 times more activity than has whole lens. This would be expected from previous studies of the enzymes in corneal epithelium (Kuhlman & Resnik, 1958).

Glutathione reductase does not seem to be one of the primary targets for radiation damage in ocular tissue (Tables 1 and 2). The decrease in glutathione reductase activity reported by Pirie *et al.* (1953) was found 3–5 months after irradiation.

iso-Citric dehydrogenase. *iso*Citric dehydrogenase of the corneal epithelium is not affected up to the fourteenth day after irradiation (Table 2). Similarly, *iso*citric dehydrogenase in the lens has been found to be unaffected by X-rays (Pirie *et al.* 1953). Although doses of radiation from 100 to 1000 r. are capable of inhibiting certain enzymes *in vitro* (Barron & Dickman, 1949), the changes produced by comparatively low levels of radiation *in vivo* are still unknown. Whatever biochemical damage was produced by 1000 r. at the intervals investigated after irradiation there was no alteration in the three enzyme systems studied even though biological injury was evident.

Certain basic differences between lens and cornea should be noted in connexion with their response to irradiation. The corneal epithelium is multilayered and has a different physiological function to perform from that of the lens epithelium. In addition, the corneal epithelium is capable of sloughing off damaged cells, which the lens epithelium cannot do. Nevertheless, histological studies of the epithelial layers of both cornea (Friedenwald & Sigelman, 1953; Friedenwald *et al.* 1948) and lens (von Sallmann, 1957) reveal a qualitatively similar alteration in mitotic activity after exposure to 1000 r.

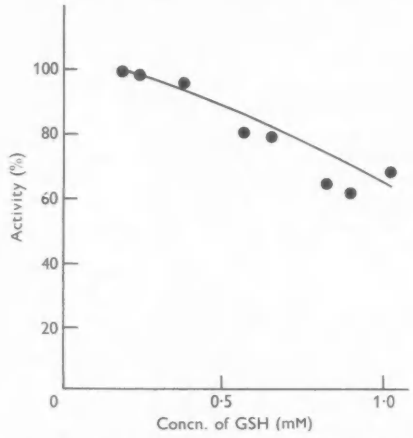


Fig. 2. Effect of GSH concentration on glutathione reductase activity of a homogenate of 200 μg. of lens. The experimental procedure for the assay of glutathione reductase described in the text was followed. GSH was added to the substrate mixture. The values of total GSH concentration indicated on the absicissa include the 2·08 μm-moles of GSH present in the 200 μg. of tissue sample.

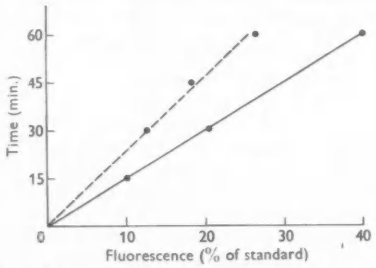


Fig. 3. Stability of glutathione reductase. ——, Brain homogenate: 35 μg. wet wt. in 50 μl. of substrate medium; all volumes were increased five times from those given in the text. Fluorescence is expressed as a percentage of the fluorescence of a solution (0·2 μg./ml.) of quinine sulphate in 0·01 N-H_2SO_4. – – –, Lens homogenate: 186 μg. wet wt. in 13 μl. of substrate medium; plotted as 0·4 times the percentage fluorescence of the quinine sulphate reference solution.

Table 3. *Glutathione reductase activity in eye tissues*

Activity is expressed as m-moles of TPN^+ formed/kg. of dry tissue/hr.

	Activity
Homogenate of whole lens of calf*	19·3
Homogenate of whole lens of rabbit*	26·7
Homogenate of whole lens of rat*	26·7
Homogenate of whole brain of rat*	1030
Rat lens†	
Capsule epithelium	188
Cortex	38
Nucleus	34
Rabbit corneal epithelium	1500

* These measurements were made on homogenates of lens and brain, and were converted into dry-weight values by assuming an average water content of 65 and 80 % for lens and brain respectively.

† Dissections from lyophilized preparations.

The cornea also differs from lens in the severity of its radiation damage in that low doses of radiation do not produce any obvious long-term changes in the cornea. In lens, on the other hand, epithelial cells migrate to the posterior part of the lens and form abnormal fibres which are the precursors of the lens opacity.

SUMMARY

1. Whole lens and cornea of rabbits exposed to X-irradiation (1000 r.) were assayed 1, 4 and 14 days afterward for hexokinase and glutathione reductase activity. Corneal epithelium was assayed for hexokinase, glutathione reductase and *iso*citric dehydrogenase 4 and 14 days after X-irradiation. In spite of morphological changes under these conditions of irradiation, there was no alteration in enzyme content.

2. A sensitive method for the assay of glutathione reductase in tissue is presented. Its activity in tissues of the rat, rabbit and calf is compared.

The authors wish to thank Dr Ludwig von Sallmann for his biomicroscopic examination of the animals, and for his advice and comments in preparing the manuscript. Also we would like to acknowledge the co-operation of Dr J. Robert Andrews for his design of a lead cone used to irradiate the animals, and Mr Henry Meyer and Mr Leo Caravaggio for their technical assistance.

REFERENCES

Barron, E. S. G. & Dickman, S. (1949). *J. gen. Physiol.* **32**, 595.

Buell, M. V., Lowry, O. H., Roberts, N. R., Chang, M. W. & Kapphahn, J. I. (1958). *J. biol. Chem.* **232**, 979.

Friedenwald, J. S., Buschke, W. & Moses, S. G. (1948). *Johns Hopk. Hosp. Bull.* **82**, 312.

Friedenwald, J. S. & Sigelman, S. (1953). *Exp. Cell Res.* **4**, 1.

Green, H., Bocher, C. A. & Leopold, I. H. (1955). *Amer. J. Ophthal.* **39**, 106.

Kuhlman, R. E. (1959). *J. cell. comp. Physiol.* (in the Press).

Kuhlman, R. E. & Resnik, R. A. (1958). *Amer. J. Ophthal.* **46**, II, 47.

Lowry, O. H. (1953). *J. Histochem. Cytochem.* **1**, 420.

Lowry, O. H., Roberts, N. R. & Kapphahn, J. I. (1957). *J. biol. Chem.* **224**, 1047.

Lowry, O. H., Roberts, N. R., Leiner, K. Y., Wu, M. & Farr, A. L. (1954). *J. biol. Chem.* **207**, 1.

Mandel, P. & Schmitt, M. L. (1956). *Experientia*, **12**, 223.

Nordmann, J. & Mandel, P. (1955). *Amer. J. Ophthal.* **40**, II, 871.

Pihl, A., Eldjarn, L. & Bremer, J. (1957). *J. biol. Chem.* **227**, 339.

Pirie, A. & van Heyningen, R. (1956). *The Biochemistry of the Eye*, p. 20. Springfield, Illinois: Charles Thomas Inc.

Pirie, A., van Heyningen, R. & Boag, J. W. (1953). *Biochem. J.* **54**, 682.

Pirie, A., van Heyningen, R. & Flanders, P. H. (1955). *Biochem. J.* **61**, 341.

Robins, E. (1957). *Exp. Cell Res.* (Suppl.), **4**, 241.

van Heyningen, R. & Pirie, A. (1953). *Biochem. J.* **53**, 436.

van Heyningen, R. & Pirie, A. (1957). *Amer. J. Ophthal.* **43**, 893.

van Heyningen, R., Pirie, A. & Boag, J. W. (1954). *Biochem. J.* **56**, 372.

von Sallmann, L. (1957). *Amer. J. Ophthal.* **44**, 159.

von Sallmann, L., Caravaggio, L., Munoz, C. M. & Drungis, A. (1957). *Amer. J. Ophthal.* **43**, 693.

von Sallmann, L., Tobias, C. A., Anger, H. O., Welch, C., Kimura, S. F., Munoz, C. M. & Drungis, A. B. (1955). *Arch. Ophthal., N.Y.*, **54**, 489.

Cerebral Metabolism in the Sheep

1. NORMAL SHEEP

By B. P. SETCHELL*

Department of Animal Pathology, University of Cambridge

(*Received* 18 *August* 1958)

The work reported in this paper was carried out to provide data for comparison with the results obtained with the brains from abnormal sheep, and to see if any difference could be found between the metabolism *in vitro* of sheep and other animals which could account for the greater resistance of the sheep to hypoglycaemia (Reid, 1951; McClymont & Setchell, 1956*a*).

* Present address: Veterinary Research Station, Glenfield, N.S.W., Australia.

EXPERIMENTAL

Preparation of reagents

Sodium pyruvate was prepared by the method of Robertson (1942). Cytochrome *c* was prepared by the method of Keilin & Hartree (1945), and the concentration of the final solution determined spectroscopically by the method of Potter (1949).

The yeast-hexokinase preparation used was stage 3*a* of the method of Berger, Slein, Colowick & Cori (1946).

All other reagents were commercial products and, when available, of A.R. grade.

Buffers

pH measurements were made with a Pye Universal pH meter standardized at pH 4·0, 7·0 and 9·2 with the buffer tablets supplied.

Phosphate buffer. Na_2HPO_4 (104 or 400 mM) was adjusted to pH 7·4 with HCl.

Tris buffer. 2-Amino-2-hydroxymethylpropane-1:3-diol (500 mM; tris) was adjusted to pH 7·4 with HCl.

Glycylglycine buffer. Glycylglycine (250 mM) was adjusted to pH 7·4 with NaOH.

Media

The pH values of all media were checked before use and adjusted to 7·4 where necessary with HCl or NaOH.

Phosphate saline. This contained 134 mM-NaCl, 5·4 mM-KCl, 10·4 mM-phosphate buffer, 1·3 mM-KH_2PO_4, 1·3 mM-$MgSO_4$, 1·3 mM-$CaCl_2$ and, where present, 10 mM-glucose.

Potassium-rich medium for the study of pyruvate metabolism. This contained 76 mM-KCl, 1·3 mM-$MgCl_2$, 1 mM-adenosine triphosphate (ATP), 40 mM-phosphate buffer and, where present, 3·3 mM-sodium pyruvate and 3·3 mM-sodium fumarate.

Sodium-rich medium. This contained 134 mM-NaCl, 5 mM-KCl, 1·3 mM-$MgSO_4$, 10 mM-phosphate buffer, 1·3 mM-KH_2PO_4, 1 mM-ATP and, where present, 3·3 mM-sodium pyruvate and 3·3 mM-sodium fumarate.

Tris saline and glycylglycine saline. These were the same as phosphate saline but with 50 mM-tris buffer or 25 mM-glycylglycine buffer respectively replacing the phosphate buffer.

Bicarbonate saline media. Bicarbonate saline (Krebs & Henseleit, 1932) was equilibrated with $O_2 + CO_2$ (95:5), and used without additions as 'no-substrate medium', with the addition of 20 mM-glucose as 'glucose medium' or with 10 mM-sodium glutamate and 20 mM-glucose as 'glucose + glutamate medium'.

Mitochondrial incubation medium. This contained 12 mM-sodium glutamate, 16 mM-Na_2HPO_4, 1·2 mM-adenylic acid, 8 mM-$MgCl_2$, 24 mM-KCl, 0·012 mM-cytochrome *c*, 24 mM-glucose and 40 mM-tris buffer.

Oxidative phosphorylation medium. This contained, in addition to the constituents of 'mitochondrial incubation medium', 10% of yeast-hexokinase preparation and 16 mM-potassium fluoride.

Analytical methods

Glucose. Glucose was determined by Nelson's (1944) method, the absorption being read at the maximum of 660 mμ, not at 520 mμ as was originally used.

Glycogen. Glycogen was estimated in brain slices by a micromodification of the method of Kerr (1936) similar to that used by LeBaron (1955). The brain slices (100–200 mg. fresh wt.) were digested in 0·5 ml. of aqueous 30% KOH soln. for 30 min. at 100° and then 1 ml. of ethanol was added to precipitate the glycogen. This was found to give more consistent recoveries than digestion with ethanolic KOH soln. The glycogen was sedimented by centrifuging for 30 min. at 3000 *g* after cooling. The precipitate was washed three times in the methanol–$CHCl_3$ mixture used by Kerr and the glycogen hydrolysed by treatment with N-H_2SO_4 (1 ml.) for 2 hr. in a boiling-water bath. The acid was neutralized with M-$NaHCO_3$ to pH 5–9, with wide-range indicator paper (British Drug Houses Ltd.) used externally. This was more convenient than neutralization with NaOH. Solid $NaHCO_3$ added in slight excess gave erratic results. The reducing sugar in the resulting solution was estimated by the method of Nelson (1944).

Lactate. Lactate was determined by the method of Barker & Summerson (1941) as modified by Speck, Moulder & Evans (1946).

Pyruvate and α-oxoglutarate. Pyruvate and α-oxoglutarate were determined by the method of Friedmann & Haugen (1943), ethyl acetate being used to extract the 2:4-dinitrophenylhydrazones. The absorption was read at 390 and 520 mμ and the proportion of the two acids determined from the ratio of these two absorptions.

Citrate. Citrate was determined by the modification by McArdle (1955) of the method of Weil-Malherbe & Bone (1949).

Acetate. Acetate was determined in incubation media as the acid, which was steam-volatile in a Markham still from a mixture of saline and potassium oxalate–oxalic acid buffer, pH 4·0. This method is based on the method of Scarisbrick (1952) for volatile fatty acids in blood, without the paraffin and the *sec.*-octyl alcohol. At this pH, lactate and pyruvate are not steam-volatile.

Phosphorus. Acid-soluble phosphorus was extracted from brain slices (about 100 mg. fresh wt.) by grinding the slices in 10% trichloroacetic acid (5 ml.). The mixture was centrifuged at 3000 *g* for 10 min. at 0–5°, sufficient under these conditions to yield a clear supernatant. Second extraction and centrifuging yielded a supernatant containing less than 5% of the phosphorus in the first extract. Centrifuging at 6000 and 20 000 *g* gave similar results. Heald (1956) found that centrifuging at 3000 *g* was insufficient to sediment all the phospholipids. Considerable amounts of phospholipids were also extracted by the second lot of trichloroacetic acid. Possibly under the conditions of the present work a coarser homogenate was produced, which was more readily sedimented, than that used by Heald.

The phosphorus in the supernatant was partitioned by the barium acetate–ethanol fractionation of LePage (1949). All manipulations were carried out either in the cold room or in tubes immersed in crushed ice. The final precipitates were dissolved in 0·1 N-HCl and phosphorus was estimated by the method of Berenblum & Chain (1938) modified by Long (1943), or latterly by the modification of Weil-Malherbe & Green (1951). The two modifications gave similar results.

Phosphorus in media, as in the oxidative phosphorylation studies, was determined by the vanadomolybdate method of Simonsen, Wertman, Westover & Mehl (1946).

Water. Water content of slices was calculated as the difference between the wet weight and the weight after drying for at least 24 hr. in a hot-air oven at 100°.

Sodium and potassium. Sodium and potassium were extracted from dried slices (10–20 mg. dry wt.) by incubation in stoppered glass tubes at room temperature for at least 48 hr. in 10 ml. of 0·1 N-HNO_3 (Whittam, 1955). The acid used was stored in polythene containers. To correct for any sodium or potassium extracted from the glass of the tubes, a blank of 10 ml. was incubated in a similar manner. Sodium and potassium were then determined, in a 1 in 10 dilution of the extract in ion-free water (also kept in a polythene container), with an EEL flame photometer. The intensity of the colour produced was compared with that

produced by a blank of ion-free water and by a standard containing 0·1 μequiv. of both sodium and potassium. No interference was found, over the range of concentrations found in tissue slices, between sodium and potassium.

Chloride. Chloride was determined in a 1 ml. sample of the 0·1 N-HNO_3 extract used for sodium and potassium determinations, by a potentiometric titration with silver and silver-amalgam electrodes (Cunningham, Kirk & Brooks, 1941; Seligson, McCormick & Sleeman, cited by Hawk, Oser & Summerson, 1954). The potential developed between the electrodes was measured after the addition of each 0·05 ml. of 0·001 N-$AgNO_3$ soln. The end point of the titration was taken as the point of inflexion of the resultant sigmoid curve.

Preparation of tissues

The sheep used were mainly animals being slaughtered at an abattoir and had been fasted overnight. The heads were removed within a few minutes of death by stunning, cutting the throat and dislocating the neck. The top of the head was skinned and the top of the skull chopped away with a hatchet. With a little practice, this could be done in about a minute without tearing the dura. The brain was lifted out with the index and second fingers after breaking down its connexions with the medulla and cranial nerves.

Pieces of brain from which mitochondria were to be prepared were cut and immersed as quickly as possible in ice-cold 0·25 M-sucrose soln., from which they were removed for weighing and homogenizing. The rest of the brain was cut into slices about 1 cm. thick and placed in small glass pots, which were then packed in crushed ice in a Thermos flask for transport to the laboratory. From death to final immersion of the pots in ice took no more than 10 min.

Guinea-pig and rabbit brains, where used for comparison, were similarly treated.

Chopped brain was prepared on a hand-operated modification (Setchell, 1957) of the chopper described by McIlwain & Buddle (1953).

Slices were cut by the method of Deutsch (1936) with a recessed microscope slide and a blade moistened with saline. When observations were made on ion movements, the slide and blade were used dry and the slices handled with fine forceps instead of being floated in a dish of saline. Slices cut in this way are referred to as dry-cut slices.

The homogenates used for the measurements of hexokinase activity were prepared with a mortar and pestle according to Long (1952).

Homogenates (20 and 10 %, w/v) for the preparation of mitochondria and other purposes were made with an all-glass Potter–Elvehjem type of homogenizer, 0·25 M-sucrose made with boiled ion-free water and neutralized with tris (250 mM) (Werkheiser & Bartley, 1957) being used.

Mitochondria were prepared from 10 % (w/v) homogenates by the method of Schneider (1948) and resuspended in neutralized 0·25 M-sucrose so that 0·5 ml. of the suspension was equivalent to approximately 500 mg. of fresh brain.

Incubation procedures

Oxygen uptake. Measurements were made with Warburg manometers and flasks, in oxygen as the atmosphere for slices and in air for chopped tissue, homogenates and mitochondria. The oxygen uptake was determined by calculating the line of best fit by the method of least squares (Mather, 1943).

Respiratory quotients. Determinations were made by the first method of Dickens & Šimer (1930), with slices or chopped tissue (about 50 mg. in 2 ml. of phosphate saline in the special flasks at 39°).

Metabolism of pyruvate. Studies were carried out with 200 mg. of chopped brain in 3 ml. of potassium-rich medium or sodium-rich medium in an atmosphere of air. Usually two flasks contained the complete system, two contained no fumarate and two neither pyruvate nor fumarate. Equilibration was for 5 min. and incubation for 30 min., during which time the oxygen uptake was measured. The reaction was stopped by adding 0·3 ml. of 80 % trichloroacetic acid from the side arm and the contents of the flask were centrifuged. Pyruvate, α-oxoglutarate, citrate, lactate and acetate were estimated in the supernatant.

Phosphocreatine resynthesis. When the tissue was used for the study of phosphocreatine resynthesis, slices (100–150 mg. fresh wt.) were incubated for 20 min. at 39° in 3 ml. of glycylglycine saline or tris saline with 10 mM-glucose in an atmosphere of oxygen.

Glycogen resynthesis. For the study of glycogen resynthesis, slices (100–200 mg. fresh wt.) were incubated for 4 hr. in 3 ml. of phosphate saline at 39°, in an atmosphere of oxygen.

Ion and water movements. For studying the movements of inorganic ions and water, slices (100–150 mg. fresh wt.) were incubated for 1 hr. in 3 ml. of bicarbonate saline medium in $O_2 + CO_2$ (95:5). At the end of the incubation, slices were freed of adherent medium by blotting with hardened filter paper (Whatman no. 50) and transferred to tared tubes for weighing, drying and extraction as described above.

Hexokinase activity. Hexokinase activity was determined by the method of Long (1952).

Cytochrome–cytochrome oxidase (indophenol oxidase) activity. The activity of this system was determined by the method of Vernon (1911) with incubation for 20 min. at 28°. After the addition of ethanol and after centrifuging, the absorption at 550 mμ of the supernatant was measured and compared with that of a similar mixture of *p*-phenylenediamine and α-naphthol in which the formation of indophenol blue had been allowed to proceed to completion spontaneously.

Mitochondrial respiration. Mitochondrial suspension (0·5 ml.) in neutralized 0·25 M-sucrose was added to 2·5 ml. of mitochondrial incubation medium. Incubation was at 39° in an atmosphere of air.

Oxidative phosphorylation. Mitochondrial suspension (0·5 ml.) or 1 ml. of 20 % homogenate in neutralized 0·25 M-sucrose was added to 2·5 ml. of oxidative phosphorylation medium. Incubation was at 39° in an atmosphere of air, usually for 15 min., when the reaction was stopped by the addition of 0·3 ml. of 80 % trichloroacetic acid from the side arm. The contents of the flask were centrifuged and inorganic phosphate was estimated in the supernatant.

Electrical stimulation of slices

The effect of electrical stimulation on the respiration of slices was studied in flasks, described by McIlwain (1951) as type *A*, with tungsten wires sealed through the glass.

The slices were mounted in Perspex–silver wire grids, type *H*, described by Ayres & McIlwain (1953). Stimulation was with 50 cyc./sec. a.c. from a transformer fed from the mains. Total current (r.m.s.) was observed continuously, and the current to the individual flasks usually at intervals of 5 min., with a rectifying moving-coil meter. One of the leads to each flask was fitted with a plug and socket which could be disconnected and plugged into the leads of the meter. Voltage was checked at the transformer at the beginning and end of each experiment.

RESULTS

Oxygen uptake and changes of the concentrations of substrate and products by sheep and other brains in the presence of various substrates

No substrate. As had been found earlier (Quastel & Wheatley, 1932), there is quite a considerable uptake of oxygen by sheep brain in the absence of substrate, but this gradually decreases with time. As a percentage of the initial rate, the decrease with time is much less than with guinea-pig brain. It is not certain whether this indicates the availability of more endogenous substrate in the sheep or whether it is simply a reflexion of the slower initial rate. The total oxygen uptake over the whole period is usually greater per gram of guinea-pig brain (Table 1).

The production of lactate by sheep brain is negligible in the absence of substrate.

Glucose. Sheep-brain slices took up glucose from saline *in vitro* and the oxygen uptake and lactate production were greater in the presence of glucose than with no substrate. The average oxygen uptake of slices of normal sheep brain in phosphate saline containing 10 mM-glucose was 46 μmoles/g. wet wt./hr. (range 36–61; 17 observations), and the production of lactate over a 90-min. period was 21 μmoles/g./hr. (range 15–26; six observations). Glucose disappeared from the medium at the rate of 26 μmoles/g./hr. (range 21–28) (six observations) measured at the end of a 90 min. period. Small amounts of keto acids, presumably pyruvate and α-oxoglutarate (1–2 μmoles/g./hr.), appeared in the medium during incubation of brain slices in glucose-containing media.

The figure for oxygen uptake is 0·75 of that for guinea-pig brain under similar conditions (63 μ-moles/g./hr.; range 58–70, 13 observations). A similar ratio for the two species was found by Krebs (1950) but the absolute rates he found for both were higher, possibly due to the inclusion in the medium he used of pyruvate, fumarate and glutamate as well as glucose.

Pyruvate. Most of the earlier work on pyruvate metabolism was done with dispersions of pigeon brain prepared in a mortar and pestle. However, the respiration of dispersions prepared in this way from sheep brain was very unreliable [Banga, Ochoa & Peters (1939) found that rat-brain dispersions were similarly unreliable], and as a method was wanted for application to individual abnormal brains, chopped brain was used in a potassium-rich medium (for details see Experimental). With this preparation, the oxygen uptake was greater in the presence of pyruvate than with no substrate, pyruvate was taken up from the medium and citrate and α-oxoglutarate appeared, all at reproducible rates. In this medium, the absence of fumarate, ATP or inorganic phosphate decreased the oxygen uptake and the pyruvate uptake; the absence of fumarate or ATP decreased the formation of α-oxoglutarate and citrate, but the absence of magnesium had no effect. In sodium-rich medium, the absence of fumarate had much less definite effects (Tables 2 and 3).

The respiration of chopped brain was greater in the potassium-rich medium than in the sodium-rich medium, although both were approximately isosmotic with plasma. This is contrary to the findings of Dickens & Greville (1935), who found an increase in respiration only if high concentrations of potassium salts were added to an already isosmotic medium. On the other hand, Dixon (1949) and Webb & Elliott (1951) found increases in respiration with high-potassium concentrations in isosmotic media.

Table 1. *Oxygen uptake of chopped brain in the absence of substrate*

	Guinea pig (five observations in three animals)	Sheep (seven observations in three animals)	*P* (Student's *t* test)
Total oxygen uptake in 5 hr. (μmoles/g. fresh wt.)	136±5	104±6	<0·01
Oxygen uptake during first hour (μmoles/g. fresh wt.)	56±3	30±2	<0·001
Oxygen uptake in succeedings hours as percentage of uptake in first hour			
Second hour	71%±5	82%±2	0·1>*P*>0·05
Third hour	40%±7	69%±3	<0·01
Fourth hour	25%±6	62%±3	<0·001
Fifth hour	13%±3	44%±3	<0·001

The preparation contained considerable quantities (3–4 μmoles/flask) of lactate, which were only slightly affected by incubation. Slight decreases were found in the flasks incubated without added pyruvate, and slight increase in those with pyruvate, but the changes were small compared with the initial concentrations.

Acetate was produced in measurable quantities by chopped brain when pyruvate and fumarate were present and the amount was increased by the absence of fumarate.

All these findings are in accord with those of Banga *et al.* (1939), Coxon, Liébecq & Peters (1949) and Coxon (1953), which were obtained with pigeon brain. The present findings, taken with those of Krebs, Eggleston, Kleinzeller & Smyth (1940), provide good evidence that the metabolism of pyruvate by the sheep brain proceeds via the tricarboxylic acid cycle.

Acetate. Acetate was not taken up from the medium by sheep-brain slices and the oxygen uptake was unaffected by its presence. This is in accordance with the findings for the sheep and other species *in vitro* (Pennington, 1952; Elliott, Grieg & Benoy, 1937; Elliott, Scott & Libet, 1942; Kleinzeller, 1943; Mann, Tennenbaum & Quastel, 1938), and for sheep brain *in vivo* (McClymont & Setchell, 1956*b*).

Inositol. Inositol had no effect on the oxygen uptake of chopped sheep brain, in accordance with the finding of Young (1936) for rat brain and not with that of Das & Guha (1935).

Respiratory quotient

The R.Q. of sheep brain *in vitro* is not affected by the presence or absence of glucose in the medium. In this regard sheep brain is similar to the other species reported (Dickens & Greville, 1933) and the findings resemble those for rabbit brain in the present work (Table 4).

Table 2. *Pyruvate metabolism by chopped sheep brain*

All rates are given in μmoles/g. fresh wt./hr. Each figure is the average of two closely agreeing values. The complete system contained 10 μmoles of sodium pyruvate, 10 μmoles of sodium fumarate, 1 μmole of ATP, 4 μmoles of $MgCl_2$, 230 μmoles of KCl and 120 μmoles of Na_2HPO_4, pH 7·4, in 3 ml.

	Complete system	No fumarate	No ATP	No magnesium	Tris instead of phosphate	No pyruvate, fumarate or ATP
Pyruvate uptake	38	31	25	39	32	—
α-Oxoglutarate production	8	3	4	9	7	0
Citrate production	2·9	0	1·3	3·1	2·9	0
Acetate production	8	14	—	—	—	0
Lactate production	10	4	2	4	10	−8
Oxygen uptake	68	55	45	73	57	51

Table 3. *Pyruvate metabolism by chopped sheep brain in potassium-rich and sodium-rich media*

All rates are given in μmoles/g. fresh wt./hr. NS, No pyruvate, fumarate or ATP. The complete system contained 10 μmoles of sodium pyruvate, 10 μmoles of sodium fumarate and 3 μmoles of ATP. Potassium-rich medium also contained 4 μmoles of $MgCl_2$, 230 μmoles of KCl and 120 μmoles of Na_2HPO_4, pH 7·4, in 3 ml. Sodium-rich medium also contained 400 μmoles of NaCl, 15 μmoles of KCl, 30 μmoles of Na_2HPO_4, pH 7·4, 4 μmoles of KH_2PO_4 and 4 μmoles of $MgSO_4$ in 3 ml.

	Pyruvate uptake		α-Oxoglutarate production			Citrate production			Oxygen uptake		
	Complete system	Fumarate absent	Complete system	Fumarate absent	NS	Complete system	Fumarate absent	NS	Complete system	Fumarate absent	NS
Potassium-rich medium	46	26	12	3	0	2·6	0·3	0	61	43	39
Sodium-rich medium	23	27	3	1	0	0·3	0·2	0	38	36	32

Table 4. *Respiratory quotient of brain* in vitro *with and without glucose*

Species	Period of experiment (hr.)	Tissue preparation	Respiratory quotient: With glucose	Respiratory quotient: Without glucose	Oxygen uptake (μmoles/flask/hr.): With glucose	Oxygen uptake (μmoles/flask/hr.): Without glucose
Sheep	2	Slices	0·94, 0·85	0·93, 0·93	5·8, 4·9	4·7, 3·7
	5	Chopped	0·97, 1·00	1·00, 1·00	2·9, 2·4	1·4, 1·4
Rabbit	1	Slices	0·85	0·95, 0·87	4·4	4·6, 3·6
	2	Slices	0·82	0·88, 0·90	6·8	6·2, 4·0

The lower values found in the shorter experiments may be due to too high a rate of oxygen uptake. Dickens (1936) pointed out a similar fault in the results of Elliott & Baker (1935), in which values even less than 0·90 were found for brain. The rate of oxygen uptake per flask was similar with or without glucose in the shorter experiments so presumably the lack of difference between the values with and without glucose is still valid.

Response to electrical stimulation

The oxygen uptake of sheep-brain slices increased when electrical stimulation (3·6 v a.c., 20–40 mA to each flask) was applied in the presence of glucose (Fig. 1). In this regard the sheep is similar to other species studied (guinea pig, rabbit, rat, human and monkey; McIlwain, 1951, 1953, 1954; McIlwain, Ayres & Forda, 1952).

However, unlike all these species, the oxygen uptake of sheep-brain slices showed a slight increase when electrical stimulation was applied in the absence of substrate. When the increase in oxygen uptake of sheep-brain slices, judged as a percentage of the initial unstimulated rate, was compared with the effect in other species, the difference was highly significant ($P < 0.001$ by Student's t test). When the effect of electrical stimulation during the second of two periods of respiration was compared, on a percentage basis, with the respiration of sheep-brain slices in the absence of substrate during two successive unstimulated periods, the difference was highly significant ($P < 0.01$).

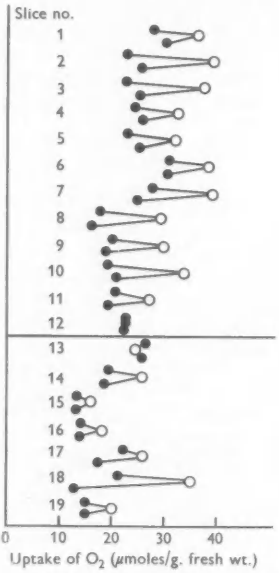


Fig. 1. Effect of electrical stimulation on the oxygen uptake of sheep-brain slices (about 100 mg. fresh wt.) in 3 ml. of phosphate saline. Each point represents the oxygen uptake/g. fresh wt./30 min. period; three points linked by a line indicate successive periods for the same slice. ●, No stimulation; ○, electrical stimulation during the 30 min. period. Slices 1–12 were incubated in phosphate saline containing 10 mM-glucose, slices 13–19 in glucose-free phosphate saline. Lactate production and glucose uptake for some of the slices during the three 30 min. periods are given in Table 5.

The reason for the increase in respiration rate in response to electrical stimulation in the absence of substrate is not known, but it could be taken as evidence for the more ready availability of some endogenous substrate.

The production of lactate by sheep-brain slices *in vitro* was also increased by electrical stimulation. The average unstimulated rate of production was 21 μmoles/g./hr. or 32 μmoles/g./90 min. period. In the flasks where electrical stimulation was applied for the second of the three 30 min. periods, the lactate production in the 90 min. period rose to about 60 μmoles/g. The actual figures for individual slices are given in Table 5. If the rate of lactate formation is similar in the two unstimulated periods to that of slices unstimulated throughout, this would mean that glycolysis during the stimulated period proceeded at a rate of about 80 μmoles/g./hr.

As exactly comparable unstimulated slices were not available for all sheep, lactate production is given as the amount in μmoles/g. produced in 90 min., comprising two unstimulated 30 min. periods and one stimulated 30 min. period.

The uptake of glucose by brain slices from the medium was increased by electrical stimulation. The uptake during the 90 min. period was increased from an average of 39 μmoles/g./90 min. when unstimulated throughout to about 60 μmoles/g./90 min. when stimulated during the second

Table 5. *Lactate production and glucose uptake by sheep-brain slices*

See under Fig. 1.

Slice no.	Lactate production (μmoles/g./90 min.)	Glucose uptake (μmoles/g./90 min.)
6	48	59
7	51	96
8	84	70
9	62	67
10	61	53
11	54	56
Mean of six unstimulated slices	32 (range 22–39)	39 (range 31–42)

30 min. period. This is equivalent to a rate of 68 μmoles/g./hr. during stimulation if calculated in the same way as the lactate production. As comparable unstimulated slices were not always available, the uptakes are given in μmoles/g./90 min., including two unstimulated 30 min. periods and one stimulated 30 min. period (Table 5).

Resynthesis of creatine phosphate

Under aerobic conditions and in the presence of glucose, sheep-brain slices resynthesized creatine phosphate. The concentrations reached in sheep-brain slices *in vitro* were very similar to those found by other workers (McIlwain, Buchel & Cheshire, 1951; Heald, 1954), and in the present work, for guinea-pig slices. The changes in inorganic phosphate were opposite to those in creatine phosphate. The results for normal sheep and guinea pig from the present work are summarized in Table 6.

Resynthesis of glycogen

Glycogen resynthesis has been demonstrated in sheep- and guinea-pig-brain slices incubated aerobically in phosphate saline with glucose, but to a smaller extent than that observed in guinea-pig-brain slices by LeBaron (1955) and McIlwain & Tresize (1956). The last-named authors showed that the levels reached *in vitro* depended on the speed of preparation of the slices after death of the animal. A possible explanation of the lower levels found in the present work in sheep- and guinea-pig-brain slices was that all preparations were made single-handed and hence probably a good deal slower than in the experiments of McIlwain & Tresize (1956) and LeBaron (1955). The concentrations found in sheep- and guinea-pig-brain slices in the present work are given in Table 7.

Glycogen concentrations in the brain in vivo

The brain-glycogen concentration, as μmoles of hexose/g. fresh wt., found in two sheep and a guinea pig killed in coma induced by the intraperitoneal injection of 200 mg. of sodium iodoacetate/kg. body wt. were 3·4 (range 3·2–3·6; three samples) for the guinea pig and 4·0 (range 3·2–4·6; six samples) and 1·9 (range 1·7–2·2; five samples) for sheep nos. 1 and 2 respectively.

Before injection of the iodoacetate, the guinea pig and sheep no. 1 were normal. Sheep no. 2 was unable to stand, though quite conscious, as poor teeth had interfered with its feeding. The undernutrition could be related to the lower concentration found in this sheep, but there is not sufficient evidence to generalize.

The concentrations found are of the same order as those found in cat, dog and rabbit brain by Kerr & Ghantus (1936); Chesler & Himwich (1943, 1944) and Ferris & Himwich (1946).

Sodium, potassium, chloride and water concentrations

When sheep-brain slices were placed in bicarbonate saline, up to 50% of the potassium was lost from the slice into the medium within 5 min. at room temperature. On incubation at 39° in bicarbonate saline containing 10 mM-glutamate and 20 mM-glucose in an atmosphere of $O_2 + CO_2$ (95:5) (glucose + glutamate medium), potassium accumulated again against the concentration gradient

Table 6. *Concentration of creatine phosphate and inorganic phosphate in incubated and unincubated brain slices*

Results are given as μmoles of P/g. fresh wt.

	Concn. of creatine phosphate		Concn. of inorganic phosphate	
Species	Unincubated	Incubated	Unincubated	Incubated
Sheep	0·6, 0·6	1·2, 1·3	4·0, 5·9	2·5, 2·0
	0·6, 0·5	1·2, 1·2	5·0, 5·0	3·0, 3·0
Guinea pig	0·5	1·3, 2·0	4·8	3·0, 3·1
	0·6, 0·6	1·2, 1·4	5·4, 5·4	3·1, 3·5

Table 7. *Concentration of glycogen in incubated and unincubated brain slices*

Results are given as μmoles of hexose/g. fresh wt.

	Time of incubation		Incubated	
Species	(hr.)	Unincubated	With glucose	Without glucose
Sheep	4	1·00, 0·50	1·16, 1·15, 1·00	0·47, 0·22
	4	0·69, 0·62	1·13, 1·11, 0·75	0·50, 0·44, 0·43
	4	0·40, 0·42	1·45, 0·81	0·34, 0·47, 0·32, 0·30
Guinea pig	4	0·64, 0·48	1·17, 1·13	

until, after incubation for 1 hr., the concentration in the slice/g. dry wt. was about the same as in dry-cut slices. In the absence of glucose and glutamate (no-substrate medium) further potassium was lost on incubation and with prolonged incubation in no-substrate medium under anaerobic conditions the potassium in the slices (per g. of tissue water) fell to a concentration only about twice that of the medium.

With glucose in the medium but no glutamate (glucose medium), some reaccumulation occurred but not as much as in glucose + glutamate medium.

Cyanide (mM) or 2:4-dinitrophenol (0·2 mM) in the medium prevented the reaccumulation of potassium by slices in glucose + glutamate medium.

When slices were incubated at 4° they contained less potassium, regardless of the medium, than slices incubated in no-substrate medium at 39° (Table 8).

When slices were placed in saline, as well as losing potassium they gained water, sodium and chloride. On subsequent incubation, however, these were not restored to the initial concentrations but increased further. Incubation in glucose medium was associated with a slightly smaller increase in water content and sodium and chloride concentrations than slices in the no-substrate medium. The water content of slices in the glucose + glutamate medium was usually intermediate between the other two but the differences were small.

There was less water in slices incubated at 4° than in those incubated at 39°, particularly in the no-substrate and the glucose + glutamate media. The sodium + potassium (total cation) concentration and the chloride concentration were also less after incubation at 4° than at 39° (Table 9).

The presence of inositol (1 mg./ml.) or glutathione (20 mM) had no effect on the water content of slices when added to glucose or glucose + glutamate media.

The potassium concentrations found after incubation were similar to those found by Terner, Eggleston & Krebs (1950) in guinea-pig brain. The results of Pappius & Elliott (1956*b*) with rat brain showed the same relative effects of glucose and glutamate but the concentrations of potassium in the incubated slices were not as high as in the dry-cut slices. One set of slices in the present work showed results similar to those of Pappius & Elliott (1956*b*), but these slices were cut much thicker than usual.

The water content of slices under the various conditions in the present work confirm the results of Elliott (1946), Stern, Eggleston, Hems & Krebs (1949), Aebi (1953) and Pappius & Elliott (1956*a*) that brain slices are not able to regulate their water content *in vitro* as efficiently as kidney and

Table 8. *Concentration of potassium in sheep-brain slices under various conditions of incubation*

Concentrations are given as m-moles/g. dry wt. Each figure is the average of two closely agreeing values.

	Cut-dry; unincubated	Incubated for 5 min. at room temp.	Incubated for 1 hr. at 39°					Incubated for 1 hr. at 4°		
			No substrate	Glucose	Glucose + glutamate	Glucose + glutamate + cyanide	Glucose + glutamate + 2:4-dinitrophenol	No substrate	Glucose	Glucose + glutamate
1	0·54	—	0·38	0·47	0·56	—	—	—	—	—
2	0·53	—	0·29	0·45	0·52	—	—	—	—	—
3	0·53	0·30	0·28	0·37	0·44	0·21	0·30	0·19	0·20	0·21
4	0·49	0·33	0·29	0·38	0·53	0·22	0·33	—	—	0·16

Table 9. *Potassium, sodium, chloride and water concentrations in sheep-brain slices after incubation for* 1 *hr. under various conditions*

Ion concentrations are given as m-moles/g. dry wt. and water in g./g. dry wt. Figures are means of groups of slices, with standard deviations.

Conditions of incubation	No. of slices in group	Concentration of ions				Concn. of water
		K^+	Na^+	$Na^+ + K^+$	Cl^-	
Cut-dry; unincubated	15	0·52±0·03	0·32±0·09	0·84±0·10	0·26±0·07	4·3±0·3
No substrate; 39°	9	0·31±0·05	0·88±0·14	1·19±0·15	0·82±0·14	7·6±0·8
Glucose; 39°	8	0·39±0·08	0·77±0·17	1·16±0·21	0·70±0·13	6·9±0·7
Glucose + glutamate; 39°	8	0·53±0·05	0·69±0·12	1·22±0·16	0·65±0·12	7·5±0·5
Glucose + glutamate; 4°	10	0·18±0·03	0·80±0·06	0·98±0·05	0·63±0·07	6·1±0·6
Glucose + glutamate + inhibitors	8	0·27±0·05	0·86±0·09	1·13±0·06	0·74±0·09	7·2±0·5

Table 10. *Uptake of oxygen and inorganic phosphate by sheep-brain mitochondria and homogenates*

All uptakes are given as μg. atoms/flask/15 min. Each flask contained mitochondria equivalent to about 500 mg. of fresh brain or homogenate equivalent to about 200 mg. of fresh brain.

	Homogenates			Mitochondria		
	Uptake of inorganic phosphate	Uptake of oxygen	P/O ratio	Uptake of inorganic phosphate	Uptake of oxygen	P/O ratio
Sheep 1	22	9·4	2·3	27	11·6	2·3
	19	11·6	1·6	29	11·8	2·5
Sheep 2	18	8·1	2·3	19	10·0	1·9
	17	8·6	2·0	22	10·0	2·2

liver slices under favourable conditions (Robinson, 1950, 1952), although slightly less swelling occurred in the presence of glucose than in its absence.

The effects of cold on the water content of incubated brain slices were similar but not as pronounced as in the results of Aebi (1953). The effect of cold is anomalous in terms of the effects on metabolism, especially as slices of other tissues swell more when incubated at lower temperatures (Robinson, 1950, 1952). Presumably, in brain slices there is a slower movement of water owing to reduced thermal diffusion at the lower temperatures.

Hexokinase activity

The hexokinase activity of 12 samples of sheep brain from four sheep was 150 μmoles of glucose phosphorylated/g. fresh wt./hr. at 30° (range 90–224), and the hexokinase activity of nine samples of guinea-pig brain from three animals was 220 μmoles of glucose phosphorylated/g./hr. (range 186–260).

These values are of the same order as those reported in the literature for other species (Long, 1952; Crane & Sols, 1953; Utter, 1950; Weil-Malherbe & Bone, 1951).

Cytochrome–cytochrome oxidase activity

The cytochrome–cytochrome oxidase activity of sheep brain was 14 μmoles of indophenol blue/g. fresh wt./hr. (range 13–16; six samples) formed

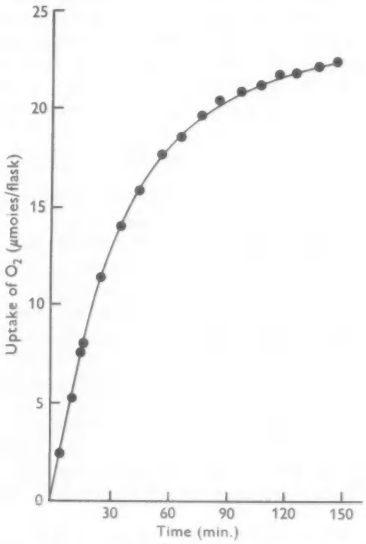


Fig. 2. Oxygen uptake by sheep-brain mitochondria (equivalent to 500 mg. of fresh brain/flask). Details of incubation and media are given in the Experimental section.

from *p*-phenylenediamine and α-naphthol at 28°. Allowing for the temperature differences, this is of the same order as the activity of sheep brain determined by Vernon (1911–12).

Oxidative phosphorylation

Homogenates of sheep brain in 0·25 M-sucrose, and sheep-brain mitochondria suspended in 0·25 M-sucrose, took up oxygen and inorganic phosphate when incubated *in vitro* in the medium described above. The P/O ratio for both preparations was about 2, which was similar to the value obtained for other species (Brody & Bain, 1952; Abood, Gerard, Banks & Tschirgi, 1952; Christie, Judah & Rees, 1953) (Table 10).

The oxygen uptake of sheep-brain mitochondria, like that of rat-brain mitochondria, fell off with time to less than 10 % of the initial rate at the end of 90 min. (Fig. 2). Unfortunately the reagents were not available for testing whether the respiration of sheep-brain mitochondria could be prolonged by the addition of cofactors shown to be effective for rat-brain mitochondria by Christie *et al.* (1953), Gallagher, Judah & Rees (1956).

DISCUSSION

A close similarity is shown between those aspects of the metabolism of the sheep brain reported in the present paper and those of other species. No difference was apparent which could account for the resistance of the sheep to hypoglycaemia, except that the oxygen uptake of sheep-brain slices, unlike those of other species, increased slightly with electrical stimulation even without added substrate.

SUMMARY

1. Some aspects of the cerebral metabolism of normal sheep have been studied.

2. Oxygen and substrate uptake and the accumulation of metabolic products were studied with slices, with glucose and pyruvate as substrates and in the absence of added substrate. Respiratory quotients were measured and the effect of electrical stimulation on the metabolism of slices was examined with glucose and without substrate.

3. The resynthesis of creatine phosphate and glycogen *in vitro*, the concentration of glycogen *in vivo* and the movement of potassium, sodium and chloride ions and of water *in vitro* were studied in slices.

4. The oxidative phosphorylation and oxygen uptake of homogenates and mitochondria and the hexokinase and cytochrome–cytochrome oxidase (indophenol oxidase) activities were measured.

5. No differences between the sheep and other species could be found which could account for the resistance of the sheep to hypoglycaemia, except that the oxygen uptake of sheep-brain slices, unlike those of other species, increased slightly with electrical stimulation even without added substrate.

REFERENCES

Abood, L. G., Gerard, R. W., Banks, J. & Tschirgi, R. D. (1952). *Amer. J. Physiol.* **168**, 728.
Aebi, H. (1953). *Helv. physiol. acta*, **2**, 96.
Ayres, P. J. W. & McIlwain, H. (1953). *Biochem. J.* **55**, 607.
Banga, I., Ochoa, S. & Peters, R. A. (1939). *Biochem. J.* **33**, 1980.
Barker, S. B. & Summerson, W. H. (1941). *J. biol. Chem.* **138**, 535.
Berenblum, I. & Chain, E. B. (1938). *Biochem. J.* **32**, 295.
Berger, L., Slein, M. W., Colowick, S. P. & Cori, C. F. (1946). *J. gen. Physiol.* **29**, 379.
Brody, T. M. & Bain, J. A. (1952). *J. biol. Chem.* **195**, 685.
Chesler, A. & Himwich, H. E. (1943). *Arch. Biochem.* **2**, 175.
Chesler, A. & Himwich, H. E. (1944). *Arch. Neurol. Psychiat., Chicago*, **52**, 114.
Christie, G. S., Judah, J. D. & Rees, K. R. (1953). *Proc. Roy. Soc.* B, **141**, 523.
Coxon, R. V. (1953). *Biochem. J.* **55**, 545.
Coxon, R. V., Liébecq, C. & Peters, R. A. (1949). *Biochem. J.* **45**, 320.
Crane, R. J. & Sols, A. (1953). *J. biol. Chem.* **203**, 273.
Cunningham, B., Kirk, P. L. & Brooks, S. C. (1941). *J. biol. Chem.* **139**, 11.
Das, N. & Guha, B. C. (1935). *Hoppe-Seyl. Z.* **231**, 157.
Deutsch, W. (1936). *J. Physiol.* **87**, 56 *P*.
Dickens, F. (1936). *Biochem. J.* **30**, 661.
Dickens, F. & Greville, G. D. (1933). *Biochem. J.* **27**, 832.
Dickens, F. & Greville, G. D. (1935). *Biochem. J.* **29**, 1468.
Dickens, F. & Šimer, F. (1930). *Biochem. J.* **24**, 905.
Dixon, K. C. (1949). *J. Physiol.* **110**, 87.
Elliott, K. A. C. (1946). *Proc. Soc. exp. Biol., N.Y.*, **63**, 234.
Elliott, K. A. C. & Baker, Z. (1935). *Biochem. J.* **29**, 2433.
Elliott, K. A. C., Grieg, M. E. & Benoy, M. P. (1937). *Biochem. J.* **31**, 1003.
Elliott, K. A. C., Scott, D. B. M. & Libet, B. (1942). *J. biol. Chem.* **146**, 251.
Ferris, S. & Himwich, H. E. (1946). *Amer. J. Physiol.* **146**, 389.
Friedmann, T. E. & Haugen, G. E. (1943). *J. biol. Chem.* **147**, 415.
Gallagher, C. H., Judah, J. D. & Rees, K. R. (1956). *Biochem. J.* **62**, 436.
Hawk, P. B., Oser, B. L. & Summerson, W. H. (1954). *Practical Physiological Chemistry*, p. 672. London: J. and A. Churchill, Ltd.
Heald, P. J. (1954). *Biochem. J.* **57**, 673.
Heald, P. J. (1956). *Biochem. J.* **63**, 243.
Keilin, D. & Hartree, E. F. (1945). *Biochem. J.* **39**, 289.
Kerr, S. E. (1936). *J. biol. Chem.* **116**, 1.
Kerr, S. E. & Ghantus, M. (1936). *J. biol. Chem.* **116**, 9.
Kleinzeller, A. (1943). *Biochem. J.* **37**, 674.
Krebs, H. A. (1950). *Biochim. biophys. Acta*, **4**, 249.
Krebs, H. A., Eggleston, L. V., Kleinzeller, A. & Smyth, D. H. (1940). *Biochem. J.* **34**, 1234.
Krebs, H. A. & Henseleit, K. (1932). *Hoppe-Seyl. Z.* **210**, 33.

LeBaron, F. N. (1955). *Biochem. J.* **61**, 80.
LePage, G. A. (1949). In *Manometric Techniques and Tissue Metabolism.* Ed. by Umbreit, W. W., Burris, R. H. & Stauffer, J. F. Minneapolis: Burgess Publishing Co.
Long, C. (1943). *Biochem. J.* **37**, 215.
Long, C. (1952). *Biochem. J.* **50**, 407.
McArdle, B. (1955). *Biochem. J.* **60**, 647.
McClymont, G. L. & Setchell, B. P. (1956*a*). *Aust. vet. J.* **32**, 97.
McClymont, G. L. & Setchell, B. P. (1956*b*). *Aust. J. biol. Sci.* **9**, 184.
McIlwain, H. (1951). *Biochem. J.* **49**, 382.
McIlwain, H. (1953). *J. Neurol. Psychiat.* **16**, 257.
McIlwain, H. (1954). *Arch. Neurol. Psychiat., Chicago*, **71**, 488.
McIlwain, H., Ayres, P. J. W. & Forda, O. (1952). *J. ment. Sci.* **98**, 265.
McIlwain, H., Buchel, C. & Cheshire, J. D. (1951). *Biochem. J.* **48**, 12.
McIlwain, H. & Buddle, H. L. (1953). *Biochem. J.* **53**, 412.
McIlwain, H. & Tresize, M. A. (1956). *Biochem. J.* **63**, 250.
Mann, P. J. G., Tennenbaum, M. & Quastel, J. H. (1938). *Biochem. J.* **32**, 243.
Mather, K. (1943). *Statistical Analysis in Biology.* London: Methuen and Co. Ltd.
Nelson, N. (1944). *J. biol. Chem.* **153**, 375.
Pappius, H. M. & Elliott, K. A. C. (1956*a*). *Canad. J. Biochem. Physiol.* **34**, 1007.
Pappius, H. M. & Elliott, K. A. C. (1956*b*). *Canad. J. Biochem. Physiol.* **34**, 1053.
Pennington, R. J. (1952). *Biochem. J.* **51**, 251.
Potter, V. R. (1949). In *Manometric Techniques and Tissue Metabolism.* Ed. by Umbreit, W. W., Burris, R. H. & Stauffer, J. F. Minneapolis. Burgess Publishing Co.
Quastel, J. H. & Wheatley, A. H. M. (1932). *Biochem. J.* **26**, 725.
Reid, R. L. (1951). *Aust. J. agric. Res.* **2**, 132.
Robertson, W. B. (1942). *Science*, **96**, 91.
Robinson, J. R. (1950). *Proc. Roy. Soc.* B, **137**, 378.
Robinson, J. R. (1952). *Proc. Roy. Soc.* B, **140**, 135.
Scarisbrick, R. (1952). *Biochem. J.* **50**, xxxiv.
Schneider, W. C. (1948). *J. biol. Chem.* **176**, 259.
Setchell, B. P. (1957). Ph.D. Thesis: University of Cambridge.
Simonsen, D. G., Wertman, M., Westover, L. M. & Mehl, J. W. (1946). *J. biol. Chem.* **166**, 747.
Speck, J. F., Moulder, J. W. & Evans, E. A. (1946). *J. biol. Chem.* **164**, 119.
Stern, J. R., Eggleston, L. V., Hems, R. & Krebs, H. A. (1949). *Biochem. J.* **44**, 410.
Terner, C., Eggleston, L. V. & Krebs, H. A. (1950). *Biochem. J.* **47**, 139.
Utter, M. E. (1950). *J. biol. Chem.* **185**, 499.
Vernon, H. M. (1911). *J. Physiol.* **42**, 402.
Vernon, H. M. (1911–12). *J. Physiol.* **43**, 96.
Webb, J. L. & Elliott, K. A. C. (1951). *J. Pharmacol.* **103**, 24.
Weil-Malherbe, H. & Bone, A. D. (1949). *Biochem. J.* **45**, 377.
Weil-Malherbe, H. & Bone, A. D. (1951). *Biochem. J.* **49**, 339.
Weil-Malherbe, H. & Green, R. H. (1951). *Biochem. J.* **49**, 286.
Werkheiser, W. C. & Bartley, W. (1957). *Biochem. J.* **66**, 79.
Whittam, R. (1955). *J. Physiol.* **128**, 65*P*.
Young, L. (1936). *Proc. Soc. exp. Biol., N.Y.*, **35**, 507.

Cerebral Metabolism in the Sheep

2. IRREVERSIBLE HYPOGLYCAEMIC COMA

By B. P. SETCHELL*
Department of Animal Pathology, University of Cambridge

(*Received* 18 *August* 1958)

Hypoglycaemia in the sheep is associated with symptoms similar to those in other animals, although hypoglycaemia may continue for up to 24 hr. in the sheep before symptoms begin. If symptoms are allowed to persist for long after a state of coma is reached, treatment by raising the blood-glucose concentration becomes ineffectual—so-called irreversible coma (McClymont & Setchell, 1956). A similar condition has been reported in man and in cats (Himwich, 1951) but is uncommon in rats (Himwich, 1951) and guinea pigs (Setchell, 1957).

* Present address: Veterinary Research Station, Glenfield, N.S.W., Australia.

Apart from some observations on arteriovenous oxygen differences and cerebral metabolic rate *in vivo* (Himwich, Bowman & Fazekas, 1940; Fazekas, Alman & Parrish, 1951), little attention has been paid to the biochemical aspects of this condition and the present work was carried out in the hopes of finding a biochemical lesion which could explain the persistence of symptoms despite normal or raised blood-glucose concentrations.

EXPERIMENTAL

Methods were those described previously (Setchell, 1959). The sheep used for studying the effects of insulin hypoglycaemia were aged (i.e. more than 5 years old) Suffolk

ewes which were given 10 units of insulin, ordinary or Ultralente (Burroughs Wellcome and Co.)/kg. subcutaneously. The sheep were fasted for 24 hr. before being given the insulin. They were killed in coma when, on the basis of previous observation (McClymont & Setchell, 1956), it was considered extremely unlikely that they would recover if the blood-glucose concentration were raised. Two sheep (11/12 and 8/1) were given glycerol *per os* (120 ml.) to increase the blood-glucose concentration (Setchell & McClymont, 1955*b*) and when no change in their condition occurred within 3 hr., they were killed by cutting the throats and dislocating the necks. The brains were removed and treated as described previously (Setchell, 1959). The seven sheep studied are designated 18/10, 12/11, 11/12, 8/1, 18/1, 11/5 and 15/5.

RESULTS

Metabolism of brain slices in glucose saline and the effects of electrical stimulation. The unstimulated oxygen uptake of brain slices from the seven sheep in irreversible coma was not markedly different from that of slices from normal brains. Some were rather lower, particularly some from sheep 8/1, but most fell within the normal range (Fig. 1).

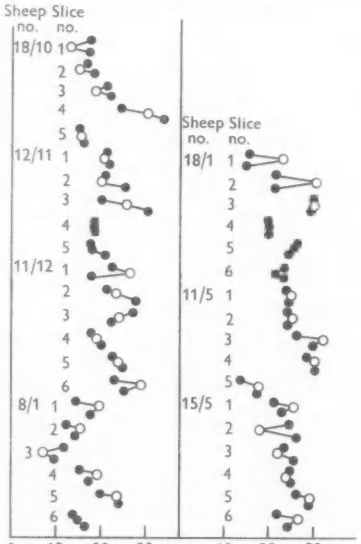


Fig. 1. Effect of electrical stimulation on the oxygen uptake of brain slices (about 100 mg. fresh wt.) from sheep in irreversible hypoglycaemic coma, incubated in 3 ml. of phosphate saline with 10 mM-glucose. Each point represents the oxygen uptake/g. fresh wt./30 min. period; three points linked by a line indicate successive periods for the same slice. ●, No stimulation; ○, electrical stimulation during the 30 min. period. Lactate production and glucose uptake for some of the slices during the three 30 min. periods are given in Table 1.

The respiration of brain slices from all insulin-treated sheep (except 18/1) showed a reduction, or absence of the increase, in response to electrical stimulation. (3·6 V a.c., 20–40 mA to each flask.) Although there was a general reduction in or an absence of the response to electrical stimulation, the degree of reduction of the response was variable in different slices from the one sheep. Some slices, in fact, showed a decreased respiration in response to electrical stimulation, and in three slices from three different sheep the rate of respiration was greater in each successive period. No explanation can be offered for these abnormal responses.

The variability of response is understandable in view of the variability of the development of the symptoms seen during hypoglycaemia and the persistence of some symptoms and not others after treatment in some cases. There seemed to be no correlation between the area from which the slices were taken and the degree of reduction in the response to electrical stimulation.

Despite the variability between slices, the effect of electrical stimulation on the oxygen uptake of slices was significantly less ($P < 0{\cdot}001$ by Student's t-test) for each of the insulin-treated sheep (except 18/1) than for the normal slices described in the previous paper, when the oxygen uptake during the period with stimulation was expressed as a percentage of the average oxygen uptake during the two periods without stimulation.

The rate of production of lactate by brain slices from sheep in irreversible coma was similar to that by brain slices from normal sheep and generally was not affected by electrical stimulation in those sheep from which some slices were unstimulated. In those sheep in which all slices were stimulated, the total lactate production over the two unstimulated periods and one stimulated period was no higher than in brain slices from other insulin-treated sheep or from normal sheep, unstimulated throughout. The lactate production for the 90 min. of the experiment is shown in Table 1.

The uptake of glucose by unstimulated brain slices from sheep in irreversible coma was somewhat higher than by slices from normal sheep. Consequently the effect of electrical stimulation could be decided only when unstimulated slices from the same sheep had been incubated. No effect of electrical stimulation could be shown in brain slices from the two insulin-treated sheep thus studied (Table 1).

Sheep 18/1 showed a normal response in lactate production and glucose uptake to electrical stimulation in two of the three slices studied. These were not the two slices which showed the normal response of respiration to electrical stimulation.

Table 1. *Lactate production and glucose uptake by sheep-brain slices*

See under Fig. 1.

Sheep no.	Slice no.	Electrical stimulation during second period	Lactate production (μmoles/g. fresh wt./90 min.)	Glucose uptake (μmoles/g. fresh wt./90 min.)
12/11	1	+	29	58
	2	+	35	54
	3	+	30	57
	4	−	31	56
	5	−	25	46
11/12	1	+	39	55
	2	+	35	65
	3	+	46	59
	4	+	40	43
	5	+	43	45
	6	+	69	49
8/1	1	+	34	39
	2	+	26	43
	3	+	25	52
	4	+	22	41
	5	+	36	37
	6	−	31	41
18/1	1	+	32	21
	2	+	60	81
	3	+	119	83
	4	−	40	57
	5	−	43	56
	6	−	49	52
11/5	1	+	26	—
	2	+	37	—
	3	+	27	—
	4	+	39	—
	5	+	38	—
15/5	1	+	32	—
	2	+	26	—
	3	+	27	—
	4	+	25	—
	5	+	36	—
	6	+	29	—
Mean of 6 normal unstimulated slices		−	32 (Range 22–39)	39 (Range 31–42)

Metabolism of pyruvate. The uptake of pyruvate and oxygen and the production of α-oxoglutarate were studied with chopped brain from the seven sheep in irreversible coma and, in some, the production of citrate, lactate and acetate was also studied. There were no differences from normal in the pyruvate or oxygen uptake with or without fumarate nor in the production of α-oxoglutarate, citrate, lactate or acetate with or without fumarate (Table 2). The oxygen uptake in the absence of substrate was slightly lower in chopped brain from insulin-treated sheep, especially when considered as a percentage of the rate in the complete medium. This is in accord with the findings of Holmes (1930), Ashford & Holmes (1931), Wortis (1935) and Elliott, Scott & Libet (1942) of a lowered rate of respiration by brain from an insulin-treated animal in the absence of substrate but not when substrate was present.

Resynthesis of creatine phosphate. The resynthesis of creatine phosphate was studied in brain slices from six of the seven animals in irreversible coma. In five of those there was no resynthesis of creatine phosphate when the slices were incubated under aerobic conditions in glucose saline (Table 3). In fact, the concentration of creatine phosphate in the incubated slices was slightly lower than in the unincubated slices, but as the concentrations involved were near the limit of sensitivity of the method this fall is not certain.

In sheep 18/1 there was resynthesis of creatine phosphate in two of the three slices studied, from an initial concentration of 0·3 μmole/g. to 0·9 μmole/g. The third incubated slice contained 0·5 μmole of creatine phosphate/g. Thus the slices from the brain of sheep 18/1 showed a normal response of respiration to electrical stimulation and a normal resynthesis of creatine phosphate.

Resynthesis of glycogen by brain slices in vitro. In brain slices from the one insulin-treated sheep studied, there was resynthesis of glycogen of the same order as that seen in normal slices (Table 4). However, as there is only a small amount of glycogen resynthesized by slices of normal brain under the present conditions, it would be possible to be certain of the significance of only extreme changes in this resynthesis.

Concentrations of potassium, sodium, chloride and water. The concentration of potassium in the slices cut without moistening with saline (dry-cut slices) was significantly lower in slices from sheep in irreversible coma. This confirms the findings of Setchell & McClymont (1955*a*).

Whereas after incubation for an hour in glucose + glutamate medium the concentration of potassium in brain slices from normal sheep was about the same as the concentration in dry-cut slices, the concentration of potassium in brain slices from the insulin-treated sheep after incubation was considerably less than in dry-cut slices from the same brain.

The concentration of potassium in brain slices from insulin-treated sheep incubated in glucose medium and in no-substrate medium was less than in brain slices from normal sheep incubated under the same conditions. There was less difference between the slices incubated in glucose medium and slices in glucose + glutamate medium when the slices were taken from an insulin-treated sheep (Table 5).

Sheep 18/1 was less affected than the other insulin-treated sheep. The potassium concentration in slices incubated in glucose + glutamate medium, although not the same as in dry-cut slices, was less reduced than in brain slices from the other insulin-treated sheep.

The water, sodium and chloride concentrations in the incubated slices were not markedly abnormal in slices from insulin-treated animals. There was less difference between the water content of slices in glucose medium and those in no-substrate medium in some insulin-treated sheep, but the large changes in water content of slices of normal brain make changes in this already small difference difficult to demonstrate.

Hexokinase activity. There was no consistent difference in the hexokinase activity between homogenates made from the brains of insulin-treated and normal sheep. There was a lowered activity in sheep 8/1, possibly associated with the lower rate of oxygen uptake seen in this sheep. The mean activity of three values for each sheep are given in Table 6. The greatest difference between the three tubes from any one sheep was 30 μmoles/g./hr.

Cytochrome–cytochrome oxidase activity. There was no difference in the cytochrome–cytochrome

Table 2. *Pyruvate metabolism by chopped brain from normal sheep and from sheep in irreversible hypoglycaemic coma*

Each figure is the average of two closely agreeing values. All rates are in μmoles/g. fresh wt./hr. (*a*) Complete system; (*b*) no fumarate; (*c*) no fumarate, pyruvate or adenosine triphosphate (ATP). Complete systems contained 10 μmoles of sodium pyruvate, 10 μmoles of sodium fumarate, 1 μmole of ATP, 4 μmoles of $MgCl_2$, 230 μmoles of KCl and 120 μmoles of Na_2HPO_4, pH 7·4, in 3 ml.

Sheep no.	Pyruvate uptake		α-Oxoglutarate production			Citrate production			Lactate production			Acetate production		Oxygen uptake			(*c*) as % of (*a*)	Slices in sodium-rich medium with glucose
	(*a*)	(*b*)	(*a*)	(*b*)	(*c*)	(*a*)	(*b*)	(*c*)	(*a*)	(*b*)	(*c*)	(*a*)	(*b*)	(*a*)	(*b*)	(*c*)		
Normal																		
1	43	33	6	2	0	2·8	1·1	0	14	8	—	—	—	65	57	51	78	48
2	38	31	8	3	0	3·3	0	0	10	4	−8	—	—	68	55	51	75	38
3	46	26	10	3	0	2·6	0·3	0	—	—	—	8	14	61	43	39	64	35
Insulin-treated																		
18/10	42	34	10	3	0	2·5	0·4	0	11	3	−6	—	—	76	60	41	54	39
12/11	34	20	6	1	0	2·1	0·5	0	—	—	—	—	—	60	39	30	50	39
11/12	38	24	7	1	0	1·7	0·2	0	—	—	—	—	—	56	26	24	43	45
												8	21					
8/1	37	28	8	2	0	2·6	0·5	0	—	—	—	—	—	59	42	31	53	30
18/1	45	32	10	3	0	5·1	0·9	0	—	—	—	—	—	69	50	41	60	51
11/5	46	34	9	5	0	—	—	—	—	—	—	6	23	54	35	27	50	48
15/5	48	41	10	4	0	—	—	—	—	—	—	8	22	56	41	35	62	50

oxidase activity between the brains of sheep in irreversible coma and normal sheep. The activity determined for five of the insulin-treated sheep is given in Table 7.

Oxidative phosphorylation. The respiration and oxidative phosphorylation of brain homogenates made from two sheep in irreversible coma was no different from that of normal sheep.

However, the mitochondria prepared in the usual way from these homogenates showed much reduced respiration and little or no associated phosphorylation. The actual extent of the phosphorylation was difficult to determine owing to the small changes involved (Table 8).

A similar state of normal oxygen and phosphate uptake by homogenate, but much reduced uptake of both oxygen and phosphate by mitochondrial preparations derived from the homogenate, could be induced by ageing a homogenate of normal brain for 2 hr. at 2° before separation of the mitochondria.

Table 3. *Creatine phosphate concentrations in brain slices from normal sheep and sheep in irreversible hypoglycaemic coma*

Concentrations are given in μmoles of P/g. fresh wt. Each figure is the average of three closely agreeing values, except for incubated slices from sheep 18/1, where the individual concentrations for each slice are given.

Sheep no.	Unincubated slices	Incubated slices
Normal		
1	0·6	1·3
2	0·6	1·2
Insulin-treated		
12/11	0·5	0·3
11/12	0·3	0·2
8/1	0·3	0·3
18/1	0·3	0·9, 0·9, 0·5
11/5	0·6	0·2
15/5	0·5	0·3

DISCUSSION

The metabolic lesion found in the brains of sheep in irreversible hypoglycaemic coma does not seem to involve the conversion of the various substrates of metabolism into their end products but rather the utilization of the energy produced from these processes, of the fundamental nature of which little is known. Further elucidation of the nature of the lesion must await advances in the knowledge of the normal mechanisms.

Table 4. *Glycogen concentration in brain slices from normal sheep and from one sheep in irreversible hypoglycaemic coma*

Concentrations are given in μmoles of hexose/g. fresh wt. Values given are averages with range and number of observations.

Sheep	Unincubated slices	Incubated slices	
		With glucose	Without glucose
Normal	0·60 (0·42–1·00; 6)	1·07 (0·75–1·45; 8)	0·39 (0·22–0·50; 9)
Insulin-treated 18/10	0·60, 0·60	0·80 (0·60–1·00; 6)	—

Table 5. *Potassium concentration in brain slices from normal sheep, and from sheep in irreversible hypoglycaemic coma, under various conditions of incubation*

Concentrations are given in m-moles/g. dry wt. Each figure is the average of at least two closely agreeing values.

Sheep no.	Slices cut dry and unincubated	Incubated slices		
		Glucose + glutamate	Glucose	No substrate
Normal				
1	0·54	0·56	0·47	0·38
2	0·53	0·52	0·45	0·29
3	0·52	0·44	0·37	0·28
4	0·49	0·53	0·38	0·29
Insulin-treated				
12/11	0·47	0·38	0·33	0·20
11/12	0·48	0·28	0·27	0·18
8/1	0·43	0·30	0·28	0·24
18/1	0·48	0·43	0·34	0·24
11/5	0·49	0·32	0·35	0·28
15/5	0·46	0·34	0·34	0·20

To relate the present findings to the effects of hypoglycaemia on the arteriovenous oxygen difference and cerebral metabolic rate *in vivo*, it must be decided whether the metabolism *in vivo* is better correlated with the unstimulated or the stimulated metabolism *in vitro*. The overall metabolism *in vivo* is probably somewhere intermediate between the stimulated and unstimulated metabolism *in vitro*, but the rate of oxygen uptake *in vivo* corresponds more closely with the stimulated rate *in vitro* (McIlwain, 1955).

If this is so, then a reduced rate of respiration with electrical stimulation should be reflected in a lower oxygen uptake *in vivo*. In other species, this is certainly the case (Himwich, 1951), but in the one sheep for which data are available (McClymont & Setchell, quoted by Setchell, 1957) no marked changes in arteriovenous oxygen difference were demonstrated during hypoglycaemia. As no estimate of cerebral blood flow was made, this finding is not unequivocal evidence and further work is necessary.

The finding of a failure in the resynthesis of creatine phosphate *in vitro* is possibly related to the findings of a lowered creatine phosphate concentration in the brains of hypoglycaemic animals *in vivo* (Olsen & Klein, 1946), although the concentration of creatine phosphate was normal in the brains of hypoglycaemic animals which had been anaesthetized (Kerr & Ghantus, 1936). Unfortunately, neither report gives the concentration of creatine phosphate in brains of animals in irreversible coma after treatment with glucose, so the changes found may have simply been a temporary reflexion of the lowered production of energy from the metabolism of glucose rather than the unavailability of the energy produced.

The lowered reaccumulation *in vitro* of potassium by brain slices from sheep in irreversible coma may be related to the lowered concentration of potassium found in the brains of sheep and cats in irreversible hypoglycaemic coma (Yannet, 1939; Setchell & McClymont, 1955*a* and the present work). In the brains of insulin-treated sheep, the concentration of potassium *in vivo*, although less than the normal concentration *in vivo*, was greater than the concentration in slices from insulin-treated sheep incubated *in vitro* in 'glucose + glutamate medium'. This is unlike normal brain, in which a concentration is maintained *in vitro* in

Table 6. *Hexokinase activity of brain from normal sheep and from sheep in irreversible hypoglycaemic coma*

Activity is given in μmoles of glucose phosphorylated/g. fresh wt./hr.

Sheep no.	Activity
Normal	156±11
18/10	150
12/11	125
11/12	174
8/1	70
18/1	178
11/5	138
15/5	146

Table 7. *Cytochrome–cytochrome oxidase activity of chopped brain from normal sheep and sheep in irreversible hypoglycaemic coma*

Activity is given in μmoles of indophenol blue formed/g. fresh wt./hr. Each figure is the average of two closely agreeing figures.

Sheep no.	Activity at 28°
Normal	
1	16
2	13
3	13
Insulin-treated	
18/10	15
12/11	12
11/12	12
8/1	>11*
18/1	15

* Slightly cloudy; the colour was compared visually with standard.

Table 8. *Oxygen and inorganic phosphate uptake by brain homogenates and mitochondria from sheep in irreversible hypoglycaemic coma*

All uptakes are given in μg. atoms/flask/15 min. Each flask contained mitochondria equivalent to about 500 mg. of fresh brain or homogenate equivalent to about 200 mg. of fresh brain.

	Homogenates			Mitochondria		
Sheep no.	Inorganic phosphate uptake	Oxygen uptake	P/O ratio	Inorganic phosphate uptake	Oxygen uptake	P/O ratio
Normal	18	8·4	2·2	21	10·0	2·1
Normal (homogenate aged for 2 hr. at 2°)	17	9·0	1·8	0	0	—
11/5	20	8·8	2·3	0	1·1	—
	15	8·0	1·9	0	0·8	—
15/5	18	8·8	2·1	2	3·0	0·7
	17	8·2	2·1	0·5	2·4	0·2

'glucose + glutamate medium' of the same order (on a dry-weight basis) as the concentration *in vivo*. Presumably this difference is due to the essentially unphysiological conditions of incubation emphasizing the difference from normal brains.

It should be emphasized that the findings in the present work, although reasonably consistent in themselves, are only of a preliminary nature. This is a result of the small numbers of sheep used and of the fact that all these animals were studied in the same stage, the early stages of irreversible coma. Nothing is known of the metabolism of brains from sheep after protracted periods of coma or those showing early symptoms. By analogy, perhaps unwise, from the brains of other animals in early coma (Holmes, 1930; Ashford & Holmes, 1931; Elliott *et al.* 1942; Wortis, 1935; Setchell, 1957), it would seem likely that the metabolism of the brains of sheep in early coma would be normal if glucose were present.

SUMMARY

1. The metabolism has been studied of the brains of sheep in irreversible hypoglycaemic coma after the injection of large doses of insulin.

2. Abnormalities were found in the response of oxygen and glucose uptakes and lactate production to electrical stimulation, in the resynthesis of creatine phosphate, in the reaccumulation of potassium ions *in vitro* and in the oxygen uptake and oxidative phosphorylation by mitochondria.

3. The metabolism of glucose and pyruvate in the absence of electrical stimulation, the hexokinase and cytochrome–cytochrome oxidase activities and the oxygen uptake and oxidative phosphorylation of homogenates were normal.

4. The implications of this 'metabolic lesion' are discussed.

I would like to thank the Walter and Eliza Hall Trust for the Walter and Eliza Hall Veterinary Science Research Fellowship of the University of Sydney, during the tenure of which this work was carried out, and the N.S.W. Department of Agriculture for leave to enable me to carry out the work.

It is a pleasure to thank Professor W. I. B. Beveridge and the members of his Department for the hospitality accorded to me by the Department during my stay in Cambridge. More especially, I would like to thank my supervisor, Mr A. A. Wilson, for his invaluable help and encouragement.

I would also like to thank Mr R. Kimberley for invaluable help with the sheep and for general assistance and to Miss Jill Mizen for part-time assistance in the early part of the work.

REFERENCES

Ashford, C. A. & Holmes, E. G. (1931). *Biochem. J.* **25**, 2028.
Elliott, K. A. C., Scott, D. B. M. & Libet, B. (1942). *J. biol. Chem.* **146**, 251.
Fazekas, J. F., Alman, R. W. & Parrish, A. E. (1951). *Amer. J. med. Sci.* **222**, 640.
Himwich, H. E. (1951). *Brain Metabolism and Cerebral Disorders.* Baltimore: Williams and Wilkins.
Himwich, H. E., Bowman, K. M. & Fazekas, J. F. (1940). *Arch. Neurol. Psychiat., Chicago*, **44**, 1098.
Holmes, E. G. (1930). *Biochem. J.* **24**, 915.
Kerr, S. E. & Ghantus, M. (1936). *J. biol. Chem.* **116**, 9.
McClymont, G. L. & Setchell, B. P. (1956). *Aust. vet. J.* **32**, 97.
McIlwain, H. (1955). *Biochemistry and the Central Nervous System.* London: J. and A. Churchill Ltd.
Olsen, N. S. & Klein, J. R. (1946). *Res. Publ. Ass. nerv. ment. Dis.* **26**, 118.
Setchell, B. P. (1957). Ph.D. Thesis: University of Cambridge.
Setchell, B. P. (1959). *Biochem. J.* **72**, 265.
Setchell, B. P. & McClymont, G. L. (1955*a*). *Nature, Lond.*, **185**, 998.
Setchell, B. P. & McClymont, G. L. (1955*b*). *Aust. Vet. J.* **31**, 204.
Wortis, S. B. (1935). *Arch. Neurol. Psychiat., Chicago*, **33**, 1022.
Yannet, H. (1939). *Arch. Neurol. Psychiat., Chicago*, **42**, 237.

Studies on Phospholipids

5. THE SEPARATION OF DINITROPHENYLATED AND METHYLATED PHOSPHOLIPIDS BY COUNTERCURRENT DISTRIBUTION*

By F. D. COLLINS†

Department of Biochemistry, John Curtin School of Medical Research, Australian National University, Canberra, A.C.T.

(*Received* 19 *September* 1958)

Collins & Wheeldon (1957) have described the preparation of *N*-2:4-dinitrophenylated and methylated phospholipids and, by the use of these derivatives, have demonstrated the presence in rat liver, ox brain and egg yolk of unknown forms of aminophospholipids in addition to phosphatidylethanolamine and phosphatidylserine and the corresponding plasmalogens. Further study of these hitherto unrecognized forms of phospholipids depended on the development of efficient means of separation and of adequate criteria of purity.

* Part 4: Collins & Wheeldon (1958).

† Present address: Department of Biochemistry, University of Melbourne, Carlton, N. 3, Victoria, Australia.

Adsorption chromatography led to some breakdown but countercurrent distribution was satisfactory. A preliminary account (Collins, 1958) describes the general procedures used, and the isolation from sheep brain of a complex phospholipid which, however, does not occur, except in traces, in liver phospholipids. The term unknown aminophospholipid will be used to describe the three phospholipids with partition coefficients (after dinitrophenylation and methylation) of 1·0, 2·5 and 8·0 in carbon tetrachloride–methanol–water. The first of these corresponds to the complex phospholipid isolated from sheep brain (Collins, 1958). The present paper describes in detail the application of this technique to the phospholipids of rat liver and sheep brain.

EXPERIMENTAL

Solvent mixtures

All solvents used were reagent grade and the following mixtures were used in the countercurrent-distribution experiments. In each case the solvent mixtures were equilibrated and equal volumes of each phase used: Aqueous 85% (v/v) ethanol–light petroleum (b.p. 40–60°), as described by Lovern (1952); aqueous 95% (v/v) ethanol–light petroleum (b.p. 100–120°); methanol–light petroleum (b.p. 100–120°); CCl_4–methanol–water (62:35:3·15, by vol.), as described by Cole, Lathe & Ruthven (1953); CCl_4–$CHCl_3$–methylene chloride–methanol–water (25:15:10:40:10, by vol.), as described by Cole *et al.* (1953).

Materials

The rat-liver and sheep-brain lipids were prepared as described by Wheeldon & Collins (1957), and were dinitrophenylated and methylated as described by Collins & Wheeldon (1957).

^{32}P-Labelled rat-liver lipids. The lipids were labelled either *in vivo* by intravenous injection of about 0·1 mc of $NaH_2{}^{32}PO_4$ in 0·2–0·5 M-phosphate buffer (pH 7·4) or *in vitro* as described by Collins & Wheeldon (1957). In every case 60 min. elapsed between the introduction of ^{32}P and the extraction of the lipids.

Dimyristoyl-lecithin. Synthetic material was supplied by La Motte Chemical Products Co.

Methods

The methods for the determination of total P, *N*-2:4-dinitrophenyl (DNP) groups and plasmalogens have been described by Wheeldon & Collins (1957), and that for the quantitative determination of choline by Wheeldon & Collins (1958). The qualitative detection of lecithin was carried out by the method of Huennekens, Hanahan & Uziel (1954). The chromatographic procedure used was described by Collins & Wheeldon (1957).

Radioactivity measurements. The procedure described by Collins & Wheeldon (1957) was used, except for the measurement of the radioactivity in the large number of fractions obtained after chromatography or countercurrent distribution. In these, the solvent was removed from each fraction by distillation *in vacuo*, the residue dissolved in 10 ml. of light petroleum and the solution counted in a liquid counter.

Fatty acids. The lipids (containing 0·8–8·0 μmoles of fatty acids) were hydrolysed by refluxing with ethanolic N-HCl for 3 hr. Ethanol and HCl were removed by heating *in vacuo*; the residue was dissolved in 10 ml. of light petroleum (b.p. 40–60°) and shaken with water to remove soluble materials. The ethanolysis resulted in a quantitative yield of the ethyl esters of the fatty acids, which were determined as described by Stern & Shapiro (1953).

Inositol. The lipid samples were hydrolysed with 6 N-HCl for 24 hr. in a sealed tube at 110° and the inositol in the aqueous portion of the hydrolysate was determined by a microbiological procedure (Beadle, 1944).

Countercurrent distribution. An all-glass countercurrent-distribution machine (Quickfit) using 25 ml. in each phase and capable of 50 transfers was used. The value of the partition coefficient (k) was estimated from the position of the tube with the maximum content of the particular fraction as described by Craig (1944).

RESULTS

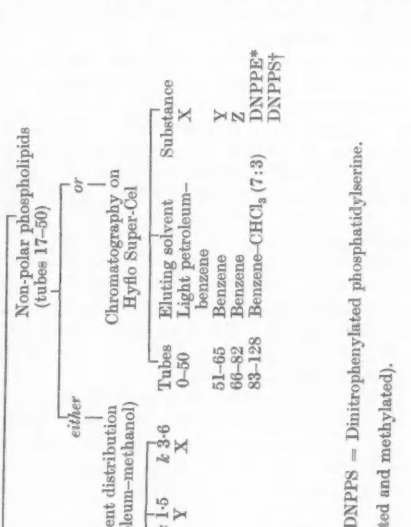
Separation of the non-polar and polar fractions

Collins & Wheeldon (1957) have described the separation of dinitrophenylated and methylated phospholipids into a 'non-polar' and a 'polar' fraction by means of a simplified countercurrent distribution with four separating funnels and a solvent system consisting of aqueous 85% (v/v) ethanol–light petroleum. The triglycerides, sterols and the methyl esters of DNP-phosphatidylethanolamine and of DNP-phosphatidylserine were preferentially soluble in the light petroleum-rich phase and constituted the non-polar fraction. The choline- and inositol-containing phospholipids and the unknown aminophospholipids (Collins & Wheeldon, 1957) were preferentially soluble in the ethanol-rich phase and made up the polar fraction.

The separation into non-polar and polar fractions has been studied in greater detail by the use of a countercurrent-distribution machine (see Fig. 1). Total lipids rather than the acetone-insoluble lipids were used to ensure that phospholipids with appreciable acetone solubility were not overlooked. The application to dinitrophenylated and methylated rat-liver lipids is shown in Fig. 2, which shows the polar fraction (tubes 1–15) and also the non-polar fraction (tubes 16–50) from which choline was absent. The material with a peak at tube 30 (k 1·5) corresponded to a mixture of phosphatidylethanolamine and phosphatidylserine as indicated by the fact that $\lambda_{max.}$ was at 328 mμ and the ratio DNP groups/P was 1·0 (Collins & Wheeldon, 1957). The small peak at tube 47 (k 16) lacked DNP groups and was designated fraction X.

When a similar experiment was carried out, with lipids labelled *in vitro* with ^{32}P, the distribution curve (Fig. 3) was similar in respect to DNP groups

Countercurrent distribution [aqueous 85% (v/v) ethanol–light petroleum; 50 transfers]



* DNPPE = Dinitrophenylated phosphatidylethanolamine. † DNPPS = Dinitrophenylated phosphatidylserine.

Fig. 1. Rat-liver and sheep-brain lipids (dinitrophenylated and methylated).

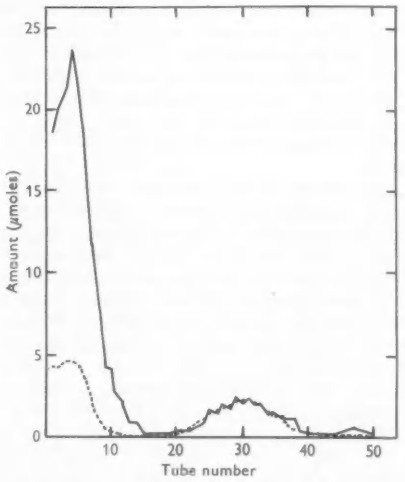


Fig. 2. Countercurrent distribution of 200 mg. of rat-liver lipids after dinitrophenylation and methylation. The solvent system was aqueous 85% (v/v) ethanol–light petroleum. Each fraction was analysed for P (——) and DNP groups (- - - -).

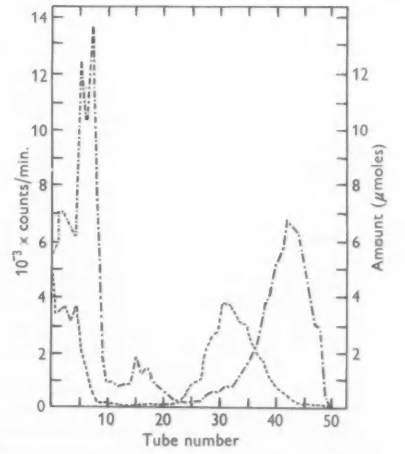


Fig. 3. Countercurrent distribution, after dinitrophenylation and methylation, of 323 mg. of rat-liver lipids which had been labelled with ^{32}P by incubation *in vitro* of a rat-liver homogenate. The solvent system was aqueous 85% (v/v) ethanol–light petroleum. Each fraction was analysed for radioactivity (—·—) and DNP groups (- - - -).

but the curve obtained by measuring the radioactivity showed two peaks which were not apparent in the first experiment (Fig. 2). The peak at tube 15 (k 0·4) would normally be divided between the polar and the non-polar fractions; that at tube 42 (k 5) was apparent only because it had a high specific radioactivity and will be referred to as fraction Y.

Examination of the non-polar fraction. These results suggested that the non-polar fraction of rat-liver phospholipids contained three phosphorus-containing subfractions. Further separation was obtained either by countercurrent distribution or by chromatography. Chromatographic separation was carried out on Hyflo Super-Cel (Fig. 4) and the eluting solvent was gradually changed from light petroleum, light petroleum–benzene mixtures, benzene and benzene–chloroform mixtures to chloroform. In the first 50 tubes there were no DNP groups but a phosphorus-containing compound was present which on countercurrent distribution proved to be similar to fraction X. The next fraction (tubes 51–65) on countercurrent distribution corresponded to fraction Y. The material in tubes 66–82 had $\lambda_{max.}$ at 410 mμ and was designated fraction Z. The material in tubes 83–128 had $\lambda_{max.}$ at 328 mμ, and the ratio DNP groups/P was 0·97, and consisted of a mixture of phosphatidylethanolamine and phosphatidylserine. Countercurrent distribution, however, did not separate this material from fraction Z.

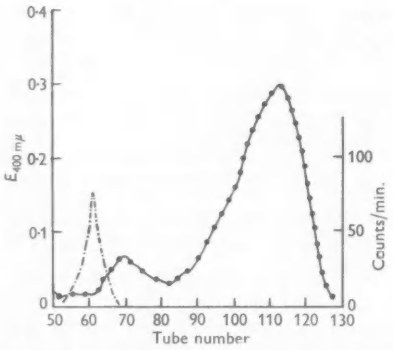


Fig. 4. Chromatography, on 50 g. of Hyflo Super-Cel, of 100 mg. of the non-polar fraction of dinitrophenylated and methylated rat-liver phospholipids which had been labelled with ^{32}P by incubation *in vitro*. Gradient elution was used, in which 500 ml. of light petroleum was added to the mixing flask and 500 ml. of benzene was added to the second flask. When the second flask had emptied, 500 ml. of chloroform was added. Each fraction (10 ml.) was analysed for radioactivity (—·—·—) and the extinction at 400 mμ (—●—) was measured in 10 ml. of light-petroleum. The numerical values are shown in Table 1.

Additional evidence for the individuality of these fractions is shown in Fig. 5, in which the non-polar fraction of rat-liver lipids, after the removal of most of the phosphatidylethanolamine and phosphatidylserine by chromatography, was distributed in methanol–light petroleum (b.p. 100–120°). The peak at tube 21 (k 0·7) corresponded to a mixture of phosphatidylethanolamine and phosphatidylserine because $\lambda_{max.}$ was at 328 mμ and the ratio DNP groups/P was 1·0. The peak at tube 30 (k 1·5) had no $\lambda_{max.}$ but a high radioactivity, and its identity with fraction Y was confirmed by measurement of k in methanol–light petroleum and in aqueous 85 % (v/v) ethanol–light petroleum. The material with a peak at tube 39 (k 3·5) also showed no $\lambda_{max.}$ and corresponded with fraction X, as shown by countercurrent distribution.

The distribution of both fractions X and Y was further investigated and the results with fraction X (Fig. 6) demonstrate that within the experimental error the radioactivity, weight and extinction at 400 mμ all follow the theoretical curve for k 3·64. The lack of selective absorption in the region 300–400 mμ confirmed the absence of DNP groups, but the fact that the extinction was proportional to P content suggested end-absorption due to a peak below 300 mμ. Although the absorption was high in this region no well-defined band was present.

A similar result (Fig. 7) was obtained with fraction Y, in that there was close agreement between the distribution of radioactivity and the theoretical curve for k 1·75, indicating the presence of a single ^{32}P-containing compound. Analysis of this material, of which 60 mg. was obtained, showed the presence of 11 μmoles of P, 43 μmoles of fatty acids and 4 μmoles of fatty aldehydes, giving a ratio of fatty acid and fatty aldehyde/P of 4·2.

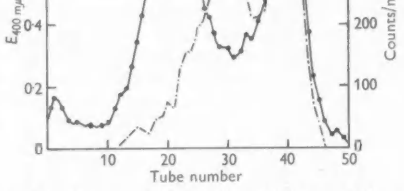


Fig. 5. Countercurrent distribution of 228 mg. of the non-polar fraction of dinitrophenylated and methylated rat-liver lipids after separation from most of the DNP-phosphatidyl-ethanolamine and -serine by chromatography. Radioactivity (—·—·—) and the extinction at 400 mμ in 10 ml. of light petroleum (—●—) were determined on each fraction.

Hydrolysis by refluxing with ethanolic 2N-KOH for 2 hr. yielded 31 mg. of unsaponifiable material, which gave a positive Liebermann–Burchard reaction, probably due to contaminating sterols because total lipids rather than the acetone-insoluble lipids had been taken.

Analysis of rat-liver lipids showed the presence of 2–3% of the minor phospholipids (fractions X, Y and Z), the properties of which were summarized by Collins (1958).

Experiments with ^{32}P *in vivo* showed that fractions Y and Z were labelled to approximately the same degree as phosphatidylethanolamine and that fraction X was somewhat less radioactive (Table 1). Labelling *in vitro*, however, resulted in a change in this relationship; thus whereas fraction Z was about twice that of phosphatidylethanolamine, both fractions X and Y, and particularly the latter, were more than ten times as radioactive.

Examination of the polar fraction. Chromatography on Hyflo Super-Cel (1 g./mg. of lipid) produced breakdown of the lipids, as indicated by the increased number of fractions obtained on rechromatography and by pronounced changes in absorption spectra. For these reasons countercurrent distribution was used and in rat-liver and sheep-brain lipids showed the presence of three fractions, containing both P and DNP groups, with characteristic absorption maxima and partition coefficients.

Rat-liver lipids (170 mg.) yielded 93 mg. of a polar fraction, with amino N/P 0·17, which was distributed in CCl_4–$CHCl_3$–methylene chloride–methanol–water (100 transfers). Two peaks in the distribution of DNP groups were obtained which corresponded to material with partition coefficients of 0·5 and 4. The tubes containing the material with k 0·5 were combined (giving 26 mg. with amino N/P 0·30) and redistributed in CCl_4–methanol–water (50 transfers). Two phosphorus-containing fractions were obtained (Fig. 8; k 0·9 and 2·5) but only one with DNP groups (k 2·5). The first fraction contained combined choline and was largely lecithin; a synthetic dimyristoyl-lecithin had

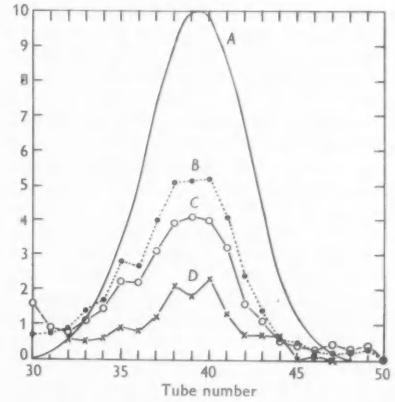


Fig. 6. Countercurrent distribution of 146 mg. of fraction X in methanol–light petroleum (b.p. 100–120°). Curve *A* is the theoretical curve calculated for k 3·64; curve *B* represents the extinction at 400 mμ in 10 ml. of light petroleum (one division = 0·1); curve *C* represents the weight of each fraction (one division = 5 mg.); curve *D* represents the radioactivity (one division = 10 counts/min.).

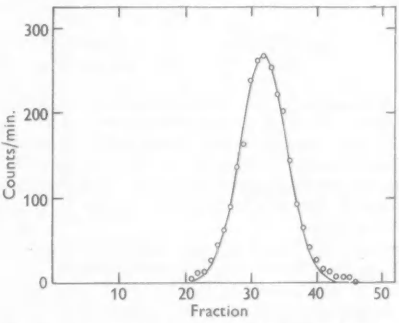


Fig. 7. Countercurrent distribution of 63 mg. of fraction Y in aqueous 95% (v/v) ethanol–light petroleum (b.p. 100–120°). The solid line has been calculated for k 1·75; the circles represent the total radioactivity in each fraction.

Table 1. *Minor phospholipids of rat liver*

Lipids were labelled either *in vivo* or *in vitro* with ^{32}P and the specific radioactivity of the total lipid was put equal to 1·0.

Fraction	X	Y	Z	Phosphatidyl-ethanolamine and -serine
Lipids labelled *in vivo*				
P as % of total lipid P	0·78	0·33	1·15	9·40
Relative specific radioactivity	0·64	2·06	1·75	1·92
Lipids labelled *in vitro* (Fig. 3)				
P as % of total lipid P	0·48	0·89	1·54	13·3
Relative specific radioactivity	2·2	11·0	0·54	0·20

k 0·9 in CCl_4–methanol–water. The second fraction (k 2·5) contained amino N and P in a ratio of 0·46 but also some lecithin, which was removed by a further 50 transfers. The overall yield of material amounted to 19 % of the DNP groups of the polar fraction and had an amino N/P 0·51. The rat-liver phospholipids had been labelled *in vitro* with ^{32}P and Fig. 8 shows that the specific radioactivity of the aminophospholipid was nearly ten times that of the lecithin.

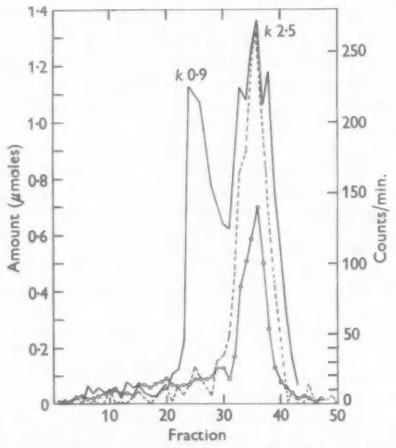


Fig. 8. Countercurrent distribution in CCl_4–methanol–water of 26 mg. of the polar fraction of rat-liver lipids after the removal of some of the lecithin in a previous countercurrent distribution in CCl_4–$CHCl_3$–methylene chloride–methanol–water (see text). Each fraction was analysed for P (——), DNP groups (—○—) and radioactivity (– – –).

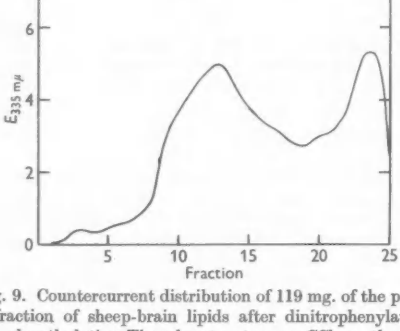


Fig. 9. Countercurrent distribution of 119 mg. of the polar fraction of sheep-brain lipids after dinitrophenylation and methylation. The solvent system was CCl_4–methanol–water and the curve represents the extinction at 335 mμ of each fraction in 10 ml. of light petroleum.

When the polar fraction (119 mg.) from sheep-brain lipids (296 mg.) was distributed in CCl_4–methanol–water two peaks in the distribution of DNP groups were obtained (Fig. 9), corresponding to partition coefficients of 1·0 and 8·0. The material with k 1·0 was free from inositol-containing phospholipids but had not been separated from the choline-containing phospholipids. Such a separation is, however, impossible to achieve in this system because of the closeness of the partition coefficient to that of lecithin (k 0·9). Separation was achieved by the use of the solubility of the material in acetone at 0°, under which conditions lecithin is insoluble. The purified material was then distributed in CCl_4–methanol–water (50 transfers), and the product (38 mg.) had a ratio of DNP groups/P equal to 0·50 and a ratio of choline/P of 0·48. Collins (1958) has given a preliminary account of further work on this material.

The material with k 8, which was present in both rat-liver and sheep-brain phospholipids, was purified by countercurrent distribution in CCl_4–methanol–water and in four distinct preparations yielded products with ratios DNP groups/P 0·70, 0·64, 0·65 and 0·66.

Amounts of up to 50 mg. of each of these fractions (k 1·0, 2·5 and 8·0) were isolated and certain of their properties determined (Collins, 1958). The ratio fatty acids/P was 0·96, 0·87 and 1·07 respectively whereas the ratio aldehyde/P was 0, 0·05 and 0 respectively.

DISCUSSION

Lovern (1952) and Cole *et al.* (1953) have applied countercurrent methods to the fractionation of lipid material from brain without obtaining any marked separation of the component phospholipids. However, the solvent systems used by these authors can be successfully applied to the fractionation of phospholipids after dinitrophenylation and methylation, which results in a reduction of the number of ionizable groups (i.e. free amino, carboxyl and phosphate groups) and in an alteration of the partition coefficients. Little or no emulsification has been experienced in the use of these solvent systems with DNP-lipids.

The application of these techniques to rat-liver and sheep-brain lipids involves a preliminary separation into polar and non-polar fractions followed by further fractionation which has resulted in the isolation of three minor phospholipids and three unknown aminophospholipids. That from sheep brain has fractions with k values 1 and 8, whilst that from rat liver has fractions with k values 2·5 and 8·0 in carbon tetrachloride–methanol–water. At present it is not possible to indicate their chemical nature but that fraction having k 8 and

isolated from rat liver would seem identical with that isolated from sheep brain, for similar values of $\lambda_{max.}$ and of the ratio DNP groups/P have been obtained.

Lecithin and the inositol-containing phospholipids have partition coefficients of 0·9 and approximately 4 respectively in carbon tetrachloride–methanol–water, and the similarity between these values and those of the aminophospholipids renders it difficult to isolate pure materials. However, small amounts have been obtained and those with k 1·0 and 2·5 had a ratio of DNP groups/P of 0·5, whereas those with k 8 had the same ratio, equal to 0·67. It may therefore be concluded that these compounds have more than one atom of P/molecule. All three compounds had a fatty acid/P ratio of 1·0.

Kennedy (1953, 1956) and Marinetti, Erbland, Albrecht & Stotz (1957*a*, *b*) have shown that, *in vitro*, [^{32}P]orthophosphate was mainly incorporated into phospholipid fractions which resembled phosphatidic acids. Marinetti *et al.* (1957*b*) have shown that there are two phosphatidic acid fractions which constituted 7% of the rat-liver lipid P and that one of these had an ester/P ratio of 6·04. In the present work fraction Y is a phosphatidic acid with an ester/P ratio of 4·2 and has a high specific radioactivity after incorporation of [^{32}P]orthophosphate *in vitro* and may correspond to one of the two fractions described by Marinetti *et al.* (1957*b*). The other fraction which has a high specific radioactivity after incorporation of [^{32}P]-orthophosphate *in vitro* is the unknown aminophospholipid, with k 2·5 (carbon tetrachloride–methanol–water), shown to be present in rat liver. This is not a phosphatidic acid, but because the ratio DNP groups/P is 0·5 there must be two P atoms per molecule, and it may be a labile compound of a phosphatidic acid and an amino N-containing phospholipid. If this were so then the phosphatidic acid component may correspond to the second fraction described by Marinetti *et al.* (1957*b*). The instability of the unknown aminophospholipids on chromatography on even such a weak adsorbent as Hyflo Super-Cel is consistent with this hypothesis.

The precise nature of fraction X remains obscure but sufficient of fraction Y was obtained to show that the ratio fatty acids/P was 4·2, which may indicate that this fraction is a bis-phosphatidic acid (see Baer, 1952; Garcia, Lovern & Olley, 1956; Olley, 1956 and Benson & Maruo, 1958).

SUMMARY

1. Countercurrent methods have been applied to the separation of dinitrophenylated and methylated phospholipids from rat liver and sheep brain.

2. Three minor phospholipids have been characterized from rat-liver lipids, two of which resemble phosphatidic acids.

3. Three different forms of unknown aminophospholipid have been distinguished by their partition coefficients (k) in carbon tetrachloride–methanol–water. Rat-liver lipids contain two with k 2·5 and 8·0 and the ratio dinitrophenyl groups/P equal to 0·5 and 0·67 respectively. Sheep-brain lipids contain phospholipids with k 1·0 and 8·0 and the ratio dinitrophenyl groups/P equal to 0·5 and 0·67 respectively, and the material with k 1·0 also contains choline (choline/P 0·48).

I wish to express my indebtedness to Mrs M. Labutis for her technical assistance and to Dr B. Holloway for his expert advice and help in establishing a culture of *Neurospora* that was both 'inositol-less' and vigorous in spore production.

REFERENCES

Baer, E. (1952). *J. biol. Chem.* **198**, 853.
Beadle, G. W. (1944). *J. biol. Chem.* **156**, 683.
Benson, A. A. & Maruo, B. (1958). *Biochim. biophys. Acta*, **27**, 189.
Cole, P. G., Lathe, G. H. & Ruthven, C. R. J. (1953). *Biochem. J.* **54**, 449.
Collins, F. D. (1958). *Nature, Lond.*, **182**, 865.
Collins, F. D. & Wheeldon, L. W. (1957). *Biochem. J.* **66**, 441.
Collins, F. D. & Wheeldon, L. W. (1958). *Biochem. J.* **70**, 46.
Craig, L. C. (1944). *J. biol. Chem.* **155**, 519.
Garcia, M. D., Lovern, J. A. & Olley, J. (1956). *Biochem. J.* **62**, 99.
Huennekens, F. M., Hanahan, D. J. & Uziel, M. (1954). *J. biol. Chem.* **206**, 443.
Kennedy, E. P. (1953). *J. biol. Chem.* **201**, 339.
Kennedy, E. P. (1956). *Canad. J. Biochem. Physiol.* **34**, 334.
Lovern, J. A. (1952). *Biochem. J.* **51**, 464.
Marinetti, G. V., Erbland, J., Albrecht, M. & Stotz, E. (1957*a*). *Biochim. biophys. Acta*, **25**, 585.
Marinetti, G. V., Erbland, J., Albrecht, M. & Stotz, E. (1957*b*). *Biochim. biophys. Acta*, **26**, 130.
Olley, J. (1956). *Biochem. J.* **62**, 107.
Stern, I. & Shapiro, B. (1953). *J. clin. Path.* **6**, 158.
Wheeldon, L. W. & Collins, F. D. (1957). *Biochem. J.* **66**, 435.
Wheeldon, L. W. & Collins, F. D. (1958). *Biochem. J.* **70**, 43.

Molecular Structure of Glycogens Isolated from the Livers of Foetal Animals

By F. L. WARREN and MARY WHITTAKER
Biochemistry Department, London Hospital Medical College, London, E. 1

(*Received* 23 *September* 1958)

Although many publications have been concerned with structural determinations of glycogen little attention has been paid to the structure of glycogen obtained from foetal tissues. Bell & Manners (1952) have recorded the β-amylolysis limit of foetal-sheep glycogen. Illingworth, Larner & Cori (1952) determined the average chain length of a sample of this glycogen, supplied by Dr D. J. Bell, and also a sample of foetal guinea-pig-liver glycogen. The other foetal glycogen which has been studied is that from pig's liver (Manners & Archibald, 1957). These authors recorded an end-group assay of this glycogen after oxidation with potassium periodate. Since investigations on foetal glycogen are so few, it was decided to make a study of its structure in samples taken throughout the gestation period. In the present work it was decided to use several methods for splitting liver glycogen in order to study any structural changes which might occur during the course of the gestation period.

The emphasis during this investigation has been on enzymic degradation and the chemical methods have been restricted solely to acid hydrolysis and periodate oxidation. In addition to the use of α-amylase, β-amylase and phosphorylase for enzymic degradation, the preparation and use of amylo-1:6-glucosidase was undertaken. This enzyme, first prepared by Cori & Larner (1951), hydrolyses the α-1:6-linkage in the phosphorylase limit dextrin, producing glucose and a dextrin which is susceptible to further phosphorylase action; the combined action of phosphorylase and amylo-1:6-glucosidase thus results in the complete digestion of glycogen with the formation of glucose 1-phosphate and glucose (Illingworth *et al.* 1952).

MATERIALS AND METHODS

Glycogen. Most of the specimens investigated were extracted from the livers obtained from foetal goats whose foetal age was known (the crown–rump length of each foetus was measured to check the age). The method of isolation, after extraction with alkali and subsequent purification, followed the outline given by Somogyi (1934). The purified glycogen was dried *in vacuo* over phosphorus pentoxide at 100°. Glycogen from other species was prepared in the same way.

α-Amylase. Freeze-dried specimens of human saliva served as the source of this enzyme.

β-Amylase. A commercial specimen of this enzyme was obtained from L. Light and Co. Ltd.

Phosphorylase. Initial preparations of this enzyme followed the method used by Illingworth & Cori (1953); this method was found difficult to reproduce and later preparations were obtained by that of Bot & Zsindely (1956).

Amylo-1:6-glucosidase. This enzyme has yet to be obtained pure. About 80% of the glucosidase present in a crude muscle extract was recovered in the precipitate obtained at 0·41 saturation with ammonium sulphate. It was obtained in a partially purified state from the pooled supernatant fluids after centrifuging the first crystals of phosphorylase (Cori, Illingworth & Keller, 1955). The mother liquor was diluted with 2·7 mM-ethylenediaminetetra-acetic acid (EDTA), pH 7·2, until the protein concentration was about 1%. Amylase was removed by means of two starch absorptions. Cori *et al.* (1955) reported that repeated starch absorptions decreased the yield of glucosidase but Larner & Schliselfeld (1956) used this stage of purification. Neutral saturated ammonium sulphate solution was then added to 0·3 saturation. The precipitate was dissolved in about half the original volume of a buffer solution containing 2·7 mM-EDTA and 3 mM-cysteine at pH 7·2. The clear solution was dialysed against this buffer at 0° for 1 hr. and then frozen in small portions before storage at −15°. The addition of glutathione (mM) restores the activity lost after repeated freezing and thawing (Larner & Schliselfeld, 1956). The enzyme was assayed by the method of Cori *et al.* (1955), whereby glucosidase is allowed to act on limit dextrin in the presence of an excess of muscle phosphorylase so that it becomes the limiting enzyme in degradation.

Acid hydrolysis. Glycogen solution (20 mg.; 1 ml.) was heated with 5 ml. of 1·5N-sulphuric acid at 100° for 2 hr. Pirt & Whelan (1951) have shown that these are the optimum conditions for minimum destruction of the glucose formed. The method of Nelson (1944) was used to estimate the glucose produced and the degree of hydrolysis was estimated on the weight of glycogen used.

Periodate oxidation. These reactions were performed in 5 ml. volumetric flasks in the refrigerator (0–5°). 0·2M-Sodium periodate solution (4 ml.) was added to an aqueous solution of glycogen (1 ml.; 50 mg.). Portions (1 ml.) were taken at suitable time intervals and run into small tubes containing ethylene glycol (0·1 ml.). All apparatus and reactants had before this stage been precooled in the refrigerator. After standing for 1 hr. at room temperature, the formic acid liberated was titrated to a methyl red end-point with 0·01N-barium hydroxide from a carbon dioxide-free microburette fitted with a hypodermic needle. Control

reactions of sodium periodate solution without the glycogen, run parallel to the oxidation, showed no increase in acidity. All glassware used in these experiments was washed in chromic acid and water followed by steaming before drying.

Periodate-oxidized glycogen was also examined by paper chromatography (Partridge, 1949) after acid hydrolysis to detect glucose, which would indicate the presence of anomalous 1:2- or 1:3-linkages in glycogen.

α-Amylolysis. An enzyme digest containing 2 ml. of glycogen solution (40 mg.), 20 ml. of phosphate–citrate buffer (0·16 M-Na_2HPO_4 solution adjusted to pH 7·0 with citric acid), 4% sodium chloride solution (1 ml.), salivary amylase solution (1 mg.; 1 ml.) and water (26 ml.) was incubated at 37°. Samples (2 ml.) were withdrawn at suitable intervals and analysed for total reducing sugar.

β-Amylolysis. These digests contained 40 mg. of glycogen solution (2 ml.), 0·2 M-acetate buffer at pH 4·6 (5 ml.), 9% sodium propionate solution (1 ml.), water (21 ml.) and β-amylase solution (1 ml.; 1 mg.) and were incubated at 28°. Samples were withdrawn at suitable intervals and the reducing-sugar content of each was determined.

Phosphorolysis. Glycogen solution (40 mg., 2 ml.) was incubated at 35° with 0·4 M-phosphate buffer (pH 6·8; 2 ml.), adenylic acid solution (1 ml.; 1 mg.), water (5 ml.) and phosphorylase solution (0·5 ml.; 9500 units/ml.). Samples (2 ml.), withdrawn at suitable intervals, were analysed for glucose 1-phosphate. The inorganic phosphate present in the reaction mixture was precipitated with magnesia mixture (2 ml.). This consisted of 0·5 M-magnesium nitrate, 2 N-ammonium nitrate and aq. 2 N-ammonia solution (Sacks, 1949). The precipitate, which removes the limit dextrins by absorption, was allowed to stand overnight at room temperature before being removed by centrifuging. The glucose 1-phosphate content of the supernatant was determined, after acid hydrolysis in N-sulphuric acid for 5 min. at 100°, as inorganic phosphate by Allen's (1940) method.

End-group determination. The reaction mixture consisted of glycogen solution (1 ml.; 20 mg.), 0·4 M-phosphate buffer (prepared from Na_2HPO_4 and KH_2PO_4) at pH 7·0 (5 ml.), 7·5 mM-EDTA at pH 7·0 (1 ml.), 1 mg. of adenylic acid (1 ml.), 9% sodium propionate solution (1 ml.), water (6 ml.), 0·1 ml. of phosphorylase (9500–14 500 units/ml.), and 0·1 ml. of glucosidase (4000 units/ml.). After incubation at 35° for 2 hr., the sum of free glucose and glucose 1-phosphate was determined by the procedure described by Cori & Larner (1951).

RESULTS

Acid hydrolysis. It was shown by paper chromatography that the sole product of acid hydrolysis of different samples of liver glycogen was glucose. The results of the chemical analyses of these hydrolytic reactions are given in Table 1.

Periodate oxidation. Since no glucose was found in the hydrolysates of solutions of periodate-oxidized glycogens it was concluded that the samples of glycogen investigated contained neither 1:2- nor 1:3-glucosidic linkages. A summary of the average chain lengths deduced from periodate oxidations of the glycogens studied is also given in Table 1, and a typical example of the course of periodate oxidation of a pooled specimen of foetal-goat-liver glycogen is illustrated in Fig. 1.

α-Amylolysis. The course of α-amylolysis was expressed as percentage conversion into maltose. The limit of such degradation is shown in Table 1.

β-Amylolysis. The β-amylolysis limits of the different glycogens examined are recorded in Table 1 as percentage conversions into maltose. The average chain lengths of all these glycogens have been determined by means of periodate oxidation (see Table 1), and so the exterior chain length for all the specimens can be calculated.

It has been shown by Peat, Whelan & Thomas (1952) that the side chains in an amylopectin β-limit dextrin contain two or three glucose residues. Summer & French (1956), after studying the action of β-amylase on model substrates, concluded that the exterior chain 'stubs' in a glycogen or amylopectin β-limit dextrin could never be shorter than 2·5 glucose units.

The exterior chain length of the glycogen specimens quoted in Table 1 have therefore been calculated on the assumption that the β-amylase action on glycogen ceased at the second or third residue from the outermost branch points. The exterior chain length by the definition of Manners (1955) is equal to the number of glucose residues removed by β-amylase plus 2·5. The interior chain length is then obtained by subtracting the exterior chain length from one less than the average number of glucose units as given in Table 1.

Phosphorolysis. Limit dextrins have been prepared from different glycogen specimens by the action of crystalline rabbit-muscle phosphorylase. The extent of phosphorolysis is recorded as the percentage conversion of glycogen into glucose 1-phosphate in Table 1.

The results obtained for phosphorolysis of foetal-goat-liver glycogen suggest a trend towards increasing degradation with increasing foetal age. This is illustrated in Fig. 2, in which age is plotted against percentage phosphorolysis.

End-group determination. The results obtained by the combined action of crystalline muscle phosphorylase and amylo-1:6-glucosidase are shown in Table 1.

Cori & Larner (1951) postulate that the ratio of free glucose to total glucose (i.e. free glucose plus phosphorylated glucose) in a digest of a polysaccharide with phosphorylase plus glucosidase should be a measure of the degree of branching and, conversely, of the average number of glucose units per branch. This has been used to calculate the average number of glucose units per branch.

An ion-exchange resin (De-Acidite FF) in the chloride form was used to separate an enzymic digest of liver glycogen after treatment with muscle

Table 1. *Structural analysis of glycogens*

Source of glycogen*	Conversion into glucose by acid (%)	$[\alpha]_D$ in water	Average chain length (periodate assay)	Action of phosphorylase + amylo-1:6-glucosidase			Action of β-amylase			Conversion by phosphorylase (%, as glucose 1-phosphate)	Conversion by α-amylase (%, as maltose)
				Glucose (%)	Free + phosphorylated glucose (%)	Average chain length	Conversion into maltose (%)	Exterior chain length	Interior chain length		
Male rabbit	95·8	202°	12·6	7·0	96·4	13·8	49·7	8–9	3–4	29·9	75·0
Female goat	98·6	200	10·8	9·1	94·0	10·3	49·7	7–8	2–3	35·9	79·3
Pregnant goat	97·5	—	12·4	7·9	95·6	12·1	53·3	9–10	1–2	32·1	75·8
Pregnant rabbit (11 days)	103·5	199	11·7	8·1	97·5	12·0	52·7	8–9	2–3	29·5	80·3
Pregnant guinea pig	—	193	—	7·9	91·4	11·5	43·2	7–8	3	27·9	—
Pregnant rabbit (25 days)	—	—	12·3	—	—	—	—	—	—	—	—
Foetal cat	99·0	200	13·1	7·9	94·5	12·0	46·4	8–9	3–4	23·7	80·5
Pregnant monkey	104·0	—	12·0	—	—	—	42·4	7–8	3–4	—	—
F.G. (pool)	95·5	198	12·3	8·8	99·0	11·3	52·3	9–10	2–3	27·0	80·7
F.G. (146)	97·5	200	12·8	8·8	96·0	11·0	52·3	8–9	2–3	46·6	80·5
F.G. (139)	94·5	202	12·6	—	—	—	51·7	9	3	43·9	79·0
F.G. (135)	94·8	—	15·7	8·4	98·3	11·7	44·0	9–10	5–6	36·8	—
F.G. (134)	95·5	—	15·3	6·8	95·3	14·0	53·3	11–12	2–3	30·2	82·5
F.G. (128)	102·0	196	11·5	7·8	94·4	12·4	50·6	8–9	2–3	28·3	80·4
F.G. (127)	—	—	12·6	6·9	98·7	14·2	49·7	8–9	3–4	30·2	77·4
F.G. (120)	101·3	—	14·2	—	—	—	48·8	9–10	3–4	25·8	83·5
F.G. (119)	—	—	12·8	7·8	98·1	12·6	—	—	—	24·5	—
F.G. (116)	94·5	203	15·3	6·8	98·9	14·6	52·7	10–11	3–4	27·1	75·3
F.G. (115)	96·3	—	12·3	7·0	96·6	13·8	—	—	—	31·1	78·8
F.G. (111)	—	—	13·8	8·1	98·5	12·2	—	—	—	28·3	—
F.G. (110)	98·5	198	14·6	6·9	93·3	13·5	51·4	10	4	29·2	81·0
F.G. (106)	—	197	12·4	8·2	98·7	12·1	51·6	8–9	2–3	25·4	80·3
F.G. (103)	—	—	12·6	—	—	—	40·0	7–8	4–5	—	—
F.G. (100+)	—	—	12·4	8·0	93·6	11·7	52·0	8–9	2–3	—	81·7
F.G. (95)	99·2	200	12·1	8·8	97·0	11·1	43·3	7–8	3–4	27·7	—

* F.G. indicates that the source of glycogen was the liver of a foetal goat of the estimated age (in days) shown in parentheses. All results are calculated in terms of weight of glycogen.

phosphorylase and glucosidase. The free glucose passed through the resin whereas the phosphorylated sugar was retained by it. A molar solution of sodium chloride was used to elute the glucose 1-phosphate. The results obtained by this procedure were in agreement with those given by the method of Cori & Larner (1951).

Specific rotations. These were measured in a semi-microtube of length 0·5 dm. with aqueous 1% (w/v) solutions of glycogen, and are shown in Table 1.

DISCUSSION

The experimental results recorded above show no marked departure from the results obtained in other structural investigations of liver glycogen. With the exception of phosphorolysis, there is no obvious variation in the behaviour of the glycogen with respect to degradation as the foetal age of the goat increases. With phosphorolysis, however, there are indications of increased susceptibility to degradation after 130 days (Fig. 2). This increased susceptibility to phosphorolysis disappears in the mature goat (Table 1). It is interesting to speculate whether the susceptibility to phosphorolysis decreases quickly after parturition.

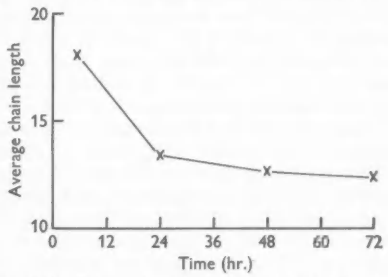


Fig. 1. Effect of varying the length of time of periodate oxidation on the calculated chain length of foetal-goat-liver glycogen. A pooled specimen of foetal-goat-liver glycogen was oxidized for different times and the degree of oxidation estimated by titration of the liberated formic acid with 0·01 N-$Ba(OH)_2$.

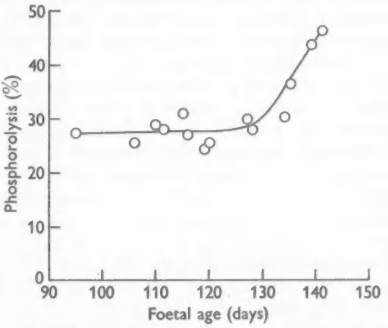


Fig. 2. Variation of the limit of phosphorolysis of foetal-goat-liver glycogen with foetal age. The degree of phosphorolysis is recorded as glucose 1-phosphate expressed as a percentage of that theoretically available. Phosphorolysis was carried out at 35° with 0·4 M-phosphate buffer (pH 6·8) for up to 4 hr.

At this stage attention may be drawn to the work of Illingworth *et al.* (1952), who found that the degree of phosphorolysis decreased progressively in glycogens isolated from the livers of embryonic, new-born and young adult guinea pigs. Unfortunately only a single specimen of embryonic liver glycogen was quoted with no indication of the approximate foetal age. These authors reported differences in structure for embryonic, new-born and young adult guinea pigs with phosphorylase degradations of 42·7, 40·8 and 39·9% respectively. The present work suggests that these figures are well within the range of normal variation for a particular age grouping of the same species.

The same authors also quote figures for the enzymically determined end group. This is measured by the free glucose formed after glycogen has been subjected to the combined actions of crystalline muscle phosphorylase and amylo-1:6-glucosidase. The figures given are 7·7, 6·9 and 6·5% of end groups in foetal, new-born and young adult liver glycogen of guinea pigs. With the same method of analysis it was found during the present investigation that the end-group content of foetal-goat-liver glycogen varies from 8·8 to 6·8% in an irregular manner within the range of foetal ages 95–146 days (full term, approx. 150 days) (Table 1). In the light of these results the conclusions drawn by Illingworth *et al.* (1952) may need modification.

A third discrepancy exists between the present work and that of Illingworth *et al.* (1952). They have shown that the values for end groups obtained by the enzymic method of phosphorylase plus glucosidase degradation showed about 20% constant deviation from the values obtained by periodate oxidation. They found that the enzymic values were the higher in all cases, but the present study has shown that the enzymic values, with few exceptions, are lower than the values derived from periodate oxidation. The arithmetic mean of the average chain lengths obtained by enzymic studies is 12·4, whereas that obtained from the mean of the periodate values is 12·9. It is suggested that amylo-1:6-glucosidase can now be prepared in a much purer state than was possible for the earlier preparations of this enzyme by Illingworth *et al.* (1952). Traces of amylases in their preparations would appreciably affect their end-group determinations and this is probably the major cause of the discrepancies.

Furthermore, Illingworth *et al.* (1952) point out that the glycogen content of such organs as liver and muscle is subject to a rapid turnover and that the composition of a particular glycogen sample depends on the previous history of the animal from which the tissue is taken. Pioneer experiments which resulted in this suggestion have been performed by Bell (1936), who determined end-group values by the methylation method of glycogen formed after ingestion of galactose by starved rabbits. Average chain lengths of 18 were found after galactose feeding. Later Bacon, Baldwin & Bell (1944) showed by the same technique that glycogen with average chain lengths of 12 was formed after the feeding of both glucose and fructose and after the injection of fructose. Feeding with sucrose was found to result in the formation of glycogen having a unit chain of 18 glucose radicals.

Schlamowitz (1951) has repeated Bell's (1936) work and attempted to find out whether the type and level of carbohydrate administered reflects itself in the production of glycogens with different branching characteristics. Glucose, galactose and fructose were administered to starved rabbits by stomach tube and by continuous intravenous infusion. Analyses for end groups by periodate oxidation on the purified liver glycogen from these animals and from normal controls showed that the values obtained after infusion of carbohydrate were greater than those obtained after administration by stomach tube. These, in turn, had end-group values greater than those for the glycogen from animals fed on rabbit pellets or sucrose or carrots. Schlamowitz considers end-group values of 19 as 'normal' whereas Bell and other workers consistently report values of 12. It is clear that the previous history of the animal studied and its feeding habits before experimentation must be taken into account.

Knowledge of the effect of anaesthetics on glycogen structure is meagre. During the present investigation many of the foetal goats and pregnant animals from which the liver glycogen was obtained were under Nembutal anaesthesia for as long as 7 hr. in some cases, but this time was not constant. There is no evidence to show whether the anaesthetic has any effect on the configuration of glycogen.

End-group assays of glycogen by periodate oxidation have been subjected to a great deal of criticism and discussion. The most recent publication on this subject is by Manners & Archibald (1957), who oxidized 15 samples of glycogen by potassium metaperiodate and also studied the method used by Potter & Hassid (1948). The latter oxidized their specimens, dissolved in sodium chloride solution, by sodium metaperiodate for 25 hr. at 2°. Manners & Archibald found that under these conditions oxidation is incomplete. Schlamowitz (1951) produced experimental evidence that oxidation under these conditions was complete. However, the average chain lengths of glycogen calculated from his results are higher than any recorded by other workers on similar specimens and suggest incomplete oxidation.

Periodate oxidations carried out during the course of this work used the conditions of Poglase, Smith & Tyler (1952). These workers used an aqueous solution and not a 1·5% sodium chloride solution of glycogen for the sodium metaperiodate oxidation at 2°. Our experimental evidence indicates that under the conditions described, the oxidation by sodium metaperiodate reached completion. Furthermore, 24 specimens of glycogen were assayed after oxidation under these conditions and, of these, 18 had average chain lengths within the range 10–14. These results agree with those of Abdel-Akher & Smith (1951), who for another 37 individual glycogens found average chain lengths of 10–14, and with those of Manners & Archibald (1957), who assayed 30 specimens of glycogen and found that the average chain lengths of 23 of these were within this range. Thus there is evidence that, under the present conditions, oxidation is complete after 72 hr. The present work is, however, in agreement with Manners & Archibald that different samples of glycogen are oxidized, under identical conditions, at slightly differing rates. The present experiments suggest that the recommendation of Manners & Archibald to continue periodate oxidation for 7–10 days is perhaps over-generous for many glycogen samples.

It is interesting to compare our results after β-amylolysis of 21 samples of glycogen with the results obtained after similar degradation by Liddle & Manners (1957*a*). These workers degraded 25 samples of glycogen, with average chain lengths varying from 8 to 18 glucose residues, with barley β-amylase. They found that the percentage conversion into maltose was 46 ± 7 in all but two instances. In a later paper Liddle & Manners (1957*b*) report average chain lengths of 7–15 in 15 samples of glycogen. β-Amylolysis during the present work has covered 21 specimens, all of which showed a percentage conversion into maltose within the range 47 ± 7. Earlier work by Bell & Manners (1952) showed that 11 glycogens (chain length about 12) on β-amylolysis with crystalline sweet-potato enzyme lost about 45% of the molecule solely as maltose. These authors also tabulate the results of β-amylolysis on 16 samples of glycogen by other workers. In this case the range of percentage conversion into maltose was 20–53 with enzyme from either wheat or barley. The β-amylase used during the present investigation was

an amorphous commercial sample, (L. Light & Co. Ltd.). The results illustrate the variation in molecular structure that exists in glycogen and this is in agreement with the work of Bell & Manners (1952) and also of Liddle & Manners (1957*a*, *b*). Afanas'eva & Stepanenko (1956) compared glycogens from rabbit and frog livers. Although the β-amylolysis limit and the point of branching were shown to be the same in both species, the frog-liver glycogen showed greater compactness in that the outer branches were shorter. In the present work this shortening of the outer chains has been observed in liver glycogens from pregnant rabbit, female goat and foetal goat.

Manners (e.g. Manners, 1954) consistently examines α-amylase digests of different glycogens (rabbit liver, brewer's yeast and human muscle from a case of von Gierke's disease) by paper chromatography with the orcinol-spray reagent (Bevenue & Williams, 1951) or the acid resorcinol reagent (Bell, 1955). In all cases he has failed to show the presence of any ketose. The α-amylase digests of liver glycogen from pregnant doe, pregnant goat and foetal goat at varying stages of gestation have been repeatedly examined by paper chromatography in this laboratory. Neither maltulose nor ketose-containing oligosaccharides could be detected. We therefore conclude, like Manners, that fructose is not a constituent of the above glycogens.

Stetten & Stetten (1954) have shown by injecting radioactive glucose intraperitoneally into rats that liver glycogen, although yielding only glucose upon hydrolysis, is metabolically inhomogeneous. They showed that the peripheral tier, determined by β-amylolysis, from liver glycogen initially accounted for the bulk of the ^{14}C. With the passage of time, however, an increasing fraction of the labelled glucose was found in the β-amylase limit dextrin until, by the twelfth hour, this fraction exceeded that in the periphery. Glycogen turnover *in vivo* is therefore not merely a replacement of pre-existing molecules by newly formed glycogen molecules. This is compatible with the inhomogeneous structures found by us and other workers.

SUMMARY

1. Glycogen was isolated from livers of goat foetuses over a wide range of foetal ages and its structure investigated chemically and enzymically.

2. Average chain lengths were calculated from the results of periodate oxidation.

3. α-Amylase, β-amylase, phosphorylase and amylo-1:6-glucosidase were employed in the enzymic degradations and average chain lengths calculated from the results of the combined actions of phosphorylase and amylo-1:6-glucosidase.

4. The average chain lengths obtained by the chemical and enzymic methods were compared.

5. The interior and exterior chain lengths were calculated from the results of β-amylolysis and periodate oxidation.

6. The 25 specimens of glycogen investigated resembled the majority of mammalian glycogens in their degree of branching.

We wish to make grateful acknowledgement to the Medical Research Council for a Scholarship awarded to one of us (M. W.) and for a grant to defray the expenses of this work.

REFERENCES

Abdel-Akher, M. & Smith, F. (1951). *J. Amer. chem. Soc.* **73**, 994.
Afanas'eva, E. M. & Stepanenko, B. N. (1956). *Biokhimiya*, **21**, 603.
Allen, R. J. L. (1940). *Biochem. J.* **34**, 858.
Bacon, J. S. D., Baldwin, E. & Bell, D. J. (1944). *Biochem. J.* **38**, 198.
Bell, D. J. (1936). *Biochem. J.* **30**, 1612.
Bell, D. J. (1955). *Modern Methods of Plant Analysis*, vol. 2, p. 21. Berlin: Springer Verlag.
Bell, D. J. & Manners, D. J. (1952). *J. chem. Soc.* p. 3641.
Bevenue, A. & Williams, E. T. (1951). *Arch. Biochem. Biophys.* **34**, 225.
Bot, G. & Zsindely, A. (1956). *Acta physiol. hung.* **9**, 73.
Cori, G. T., Illingworth, B. & Keller, P. (1955). *Methods in Enzymology*, vol. 1, p. 200. New York: Academic Press Inc.
Cori, G. T. & Larner, J. (1951). *J. biol. Chem.* **188**, 17.
Illingworth, B. & Cori, G. T. (1953). *Biochem. Prep.* **3**, 1. New York: John Wiley and Sons Inc.
Illingworth, B., Larner, J. & Cori, G. T. (1952). *J. biol. Chem.* **199**, 631.
Larner, J. & Schliselfeld, L. H. (1956). *Biochim. biophys. Acta.* **20**, 53.
Liddle, A. M. & Manners, D. J. (1957*a*). *J. chem. Soc.* p. 3432.
Liddle, A. M. & Manners, D. J. (1957*b*). *J. chem. Soc.* p. 4708.
Manners, D. J. (1954). *J. chem. Soc.* p. 3527.
Manners, D. J. (1955). *Quart. Rev. chem. Soc., Lond.*, **9**, no. 1, 73.
Manners, D. J. & Archibald, A. R. (1957). *J. chem. Soc.* p. 2205.
Nelson, N. (1944). *J. biol. Chem.* **153**, 375.
Partridge, S. M. (1949). *Nature, Lond.*, **164**, 443.
Peat, S., Whelan, W. J. & Thomas, G. J. (1952). *J. chem. Soc.* p. 4546.
Pirt, S. J. & Whelan, W. J. (1951). *J. Sci. Fd Agric.* **2**, 224.
Poglase, W. J., Smith, E. L. & Tyler, F. H. (1952). *J. biol. Chem.* **199**, 97.
Potter, A. L. & Hassid, W. Z. (1948). *J. Amer. chem. Soc.* **70**, 3488.
Sacks, J. (1949). *J. biol. Chem.* **181**, 655.
Schlamowitz, M. (1951). *J. biol. Chem.* **188**, 145.
Somogyi, M. (1934). *J. biol. Chem.* **104**, 245.
Stetten, M. R. & Stetten, D. (1954). *J. biol. Chem.* **207**, 331.
Summer, R. & French, D. (1956). *J. biol. Chem.* **222**, 469.

The Occurrence of Methylated Guanines in Ribonucleic Acids from Several Sources

By J. D. SMITH and D. B. DUNN
Agricultural Research Council Virus Research Unit, Huntingdon Road, Cambridge

(*Received* 27 *November* 1958)

A number of additional purines and pyrimidines have recently been described as minor constituents of ribonucleic acids. Cohn (1957, 1958, 1959) and Davis & Allen (1957) have described an unusual pyrimidine nucleotide from yeast ribonucleic acid, thymine, 2-methyladenine, 6-methylaminopurine and 6-dimethylaminopurine have been identified as components of ribonucleic acid from several sources (Littlefield & Dunn, 1958*a*, *b*), and 5-methylcytosine has been detected in ribonucleic acid from *Escherichia coli* (Amos & Korn, 1958) and from wheat embryo, liver and *Aerobacter aerogenes* (Dunn, unpublished work). The occurrence of methylated adenines in ribonucleic acid led us to look for methyl-substituted guanines. We have found 1-methylguanine, 6-hydroxy-2-methylaminopurine and 2-dimethylamino-6-hydroxypurine to be constituents of ribonucleic acid from several different biological sources, by identification of the free bases, ribonucleosides and ribonucleotides from the ribonucleic acids. While this work was in progress Adler, Weissmann & Gutman (1958) reported the isolation of two of these bases, 1-methylguanine and 6-hydroxy-2-methylaminopurine from an acid hydrolysate of yeast ribonucleic acid.

Preliminary results of our work have been reported (Dunn & Smith, 1959*a*, *b*).

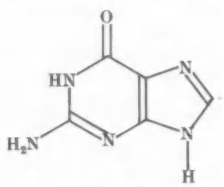
MATERIALS AND METHODS

Ribonucleic acid preparations

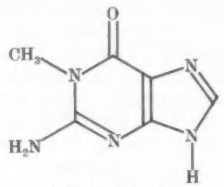
The isolation of ribonucleic acid (RNA) from *A. aerogenes*, wheat-embryo and rat-liver microsome particles has been described previously (Littlefield & Dunn, 1958*b*). The soluble RNA fraction from rat liver (Hoagland, Stephenson, Scott, Hecht & Zamecnik, 1958) was a gift from Dr M. B. Hoagland.

Plant leaf ribonucleic acid. RNA was prepared from *Nicotiana glutinosa* leaves according to Markham (1955). *Beta vulgaris* leaf RNA was given by Dr A. R. Trim. After extraction of the leaves with acetone, the RNA had been extracted with aqueous NaCl soln. at 100°. It was purified by precipitation with Al^{3+} ions (A. R. Trim, unpublished work).

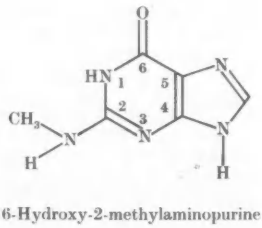
Virus ribonucleic acid. Tobacco-mosaic virus was grown in *Nicotiana tabacum* var. white Burley and was purified by differential centrifuging after treatment with 0·1 M-sodium citrate (pH 7·0) to remove any non-viral nucleoprotein (Ginoza, Atkinson & Wildman, 1954). The RNA was isolated according to Markham & Smith (1950). Turnip-yellow-mosaic virus was grown in Chinese cabbage (*Brassica chinensis*) and purified by the method of Markham



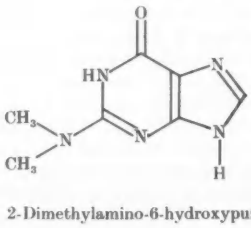
Guanine



1-Methylguanine



6-Hydroxy-2-methylaminopurine



2-Dimethylamino-6-hydroxypurine

& K. M. Smith (1949). The RNA was isolated after denaturation of the virus with ethanol (Markham & Smith, 1951).

Hydrolysis of nucleic acids

RNA was hydrolysed to nucleoside 2′- and 3′-phosphates by treatment with N-KOH at 30° for 18 hr. followed by precipitation of potassium with perchloric acid, as described by Littlefield & Dunn (1958*b*). Hydrolysis of RNA to nucleosides by Russell-viper venom, and the conversion of nucleotides into nucleosides with prostate phosphomonoesterase were both also carried out under the conditions described by Littlefield & Dunn (1958*b*). Purines were liberated from their nucleotides and nucleosides by hydrolysis in N-HCl at 100° for 1 hr. (Smith & Markham, 1950).

Paper chromatography and electrophoresis

The following solvent systems were used in chromatography: (1) Propan-2-ol (680 ml.), 11·6N-HCl (176 ml.), water to 1 l. (Wyatt, 1951). (2) Propan-2-ol (700 ml.), water (300 ml.) with NH_3 in vapour phase (Markham & Smith, 1952). (3) Butan-1-ol (770 ml.), water (130 ml.), 98% formic acid (100 ml.) (Markham & J. D. Smith, 1949). (4) Butan-1-ol (860 ml.), water (140 ml.) with NH_3 in vapour phase (Markham & J. D. Smith, 1949). (5) Water saturated with $(NH_4)_2SO_4$ (800 ml.), M-sodium acetate (180 ml.), propan-2-ol (20 ml.) (Markham & Smith, 1952).

Paper electrophoresis was carried out according to Markham & Smith (1952).

Buffers

Phosphate buffers (0·05M; pH 2·1–2·5) were prepared from 0·05M-KH_2PO_4 and 0·05M-H_3PO_4. Glycine buffer (0·05M; pH 10) was made by adding KOH to an aqueous glycine solution. Borate buffer (pH 9·2) was 0·05M-sodium tetraborate. The buffer solutions were adjusted to the correct pH by using a glass-electrode pH meter.

Spectrophotometry

Compounds located according to Markham & J. D. Smith (1949) were eluted in 0·1N-HCl and the spectra measured against those of eluates from appropriate paper blanks in a Unicam SP. 500 spectrophotometer. Alkaline spectra were measured on the same solutions after addition of 2N-KOH to give a final concentration of 0·1N-KOH.

The molar extinction coefficients of the methylated guanosines were not available. For the approximate estimation of the compounds we used the following values at pH 1·0: 6-hydroxy-2-methylaminopurine riboside, $13{\cdot}1 \times 10^3$ at 258 mμ; 2-dimethylamino-6-hydroxypurine riboside, $15{\cdot}1 \times 10^3$ at 265 mμ. These were estimated from the molar extinction coefficients of the bases (Elion, Lange & Hitchings, 1956) and the relative difference between those of guanine and guanosine. The value for 1-methylguanosine was taken as $12{\cdot}2 \times 10^3$ at 258 mμ, which is the molar extinction coefficient of guanosine. The molar extinction coefficient of uridine at pH 1·0 was taken as $10{\cdot}1 \times 10^3$ at 262 mμ.

Methylated guanines and their ribosides

Synthetic samples of 1-methylguanine, 6-hydroxy-2-methylaminopurine, 2-dimethylamino-6-hydroxypurine and 2-ethylamino-6-hydroxypurine were gifts from Dr G. H. Hitchings of the Wellcome Laboratories, Tuckahoe, N.Y., U.S.A. Another sample of 1-methylguanine was obtained from Professor D. Keilin, F.R.S. The methylamino- and dimethylamino-guanine derivatives contained traces of ultraviolet-absorbing impurities which were removed by chromatography before measurement of their ultraviolet-absorption spectra. Small quantities of guanine were identified as an impurity in the samples of 1-methylguanine. This was removed by chromatography in solvent 2 before the material was used for spectroscopy and preparation of the nucleoside.

The ribonucleosides of the methylated guanines were synthesized enzymically with an enzyme preparation from *E. coli* (Paege & Schlenk, 1952) and conditions similar to those of Littlefield & Dunn (1958*b*). To obtain sufficiently high concentrations of the methylated guanines it was necessary to dissolve them in 0·1N-HCl before adding them to the solution of inosine in phosphate buffer (Littlefield & Dunn, 1958*b*) and to adjust the solution to pH 7·0 immediately before adding the enzyme. As previously described, the products were separated in solvent 4 but the methylated guanosines did not separate readily from hypoxanthine. The material in this band was therefore chromatographed in solvent 3 where hypoxanthine moved ahead of the methylated guanosines.

RESULTS

Separation of mononucleotides

The presence of the methyl groups in 1-methylguanine, 6-hydroxy-2-methylaminopurine and 2-dimethylamino-6-hydroxypurine gave R_F values for these compounds, their nucleosides and nucleotides which were greater than those of the corresponding guanine derivatives, when chromatographed in solvent mixtures containing propan-2-ol and butan-1-ol. This behaviour, which was also observed with methylated adenines (Littlefield & Dunn, 1958*b*), was used in their separation. In addition, like guanine, the methylated guanines and their derivatives showed a blue fluorescence in ultraviolet light (260 mμ) at acid pH which aided their detection by the method of Markham & Smith (1950). 2-Dimethylamino-6-hydroxypurine and its derivatives showed a stronger fluorescence than the other two compounds.

The RNA was hydrolysed in N-KOH to give the 2′- and 3′-mononucleotides and the hydrolysate placed in a band across a sheet of Whatman no. 3 MM. filter paper and chromatographed in solvent 2. This solvent separates the four major nucleotides into two bands, one containing guanylic acid, and the other, a faster-moving band of adenylic acid, cytidylic acid and uridylic acid. The nucleotides of the three methylated guanines are found in the latter band. The material from this was eluted in water and separated by paper electrophoresis in 0·05M-phosphate buffer, pH 2·1 or, in some cases, pH 2·5. In this pH range adenylic acid and cytidylic acid have almost zero net charge and

move only slightly towards the anode, whereas uridylic acid has one negative charge. When an alkaline hydrolysate of wheat-embryo RNA was separated in this way two additional ultraviolet-absorbing bands, 1 and 2 in order of increasing mobilities, were observed (Table 1). The mobilities of these were less than that of guanylic acid. Bands 1 and 2 were eluted and re-chromatographed in solvent 2 to remove phosphate.

Identification of the methylated guanine derivatives

2-Dimethylamino-6-hydroxypurine. Band 1 contained a single substance which was identified as the ribonucleotide of 2-dimethylamino-6-hydroxypurine. After treatment with prostate phosphomonoesterase it was converted into a substance with an increased R_F value in solvent 2 corresponding to that of a nucleoside. On paper chromatography in solvents 1–4 (Table 2) and on electrophoresis in 0·05 M-borate, pH 9·2, the dephosphorylated product migrated in the same position as the enzymically synthesized 2-dimethylamino-6-hydroxypurine riboside (Table 3). Its ultraviolet-absorption spectra at pH 1·0 and pH 13·0 were the same as those of the reference nucleoside (Fig. 1; Table 4).

On hydrolysis in N-HCl at 100° for 1 hr. the nucleoside obtained from band 1 yielded a substance identified as 2-dimethylamino-6-hydroxypurine by chromatography in solvents 1–4 (Table 2) and from its ultraviolet-absorption spectra (Fig. 2; Table 4).

6-Hydroxy-2-methylaminopurine and 1-methylguanine. Band 2 from the electrophoretic separation of the nucleotides at pH 2·1 was found to be a mixture of the nucleotides of 6-hydroxy-2-methylaminopurine and 1-methylguanine. These were separated after conversion into the nucleosides since we failed to find a system for resolving the two nucleotides. The material from band 2 was incubated with prostate phosphomonoesterase. On electrophoresis in 0·05 M-borate, pH 9·2, the digest gave two ultraviolet-absorbing spots. The more

Table 1. *Electrophoretic mobilities of methylated guanine nucleotides in 0·05 M-phosphate buffer,* pH 2·1

	Mobility (cm./hr.) towards the anode at 20 v/cm.
Adenosine 3′-phosphate	1·0
Cytidine 3′-phosphate	1·0
2-Dimethylamino-6-hydroxypurine riboside 2′- and 3′-phosphates	2·6
6-Hydroxy-2-methylaminopurine riboside 2′- and 3′-phosphates	2·9
1-Methylguanosine 2′- and 3′-phosphates	2·9
Guanosine 3′-phosphate	3·4
Uridine 3′-phosphate	7·4

Table 2. *R_F values of methylated guanines and their ribosides*

Solvents: (1) propan-2-ol (680 ml.), 11·6 N-HCl (176 ml.), water to 1 l.; (2) propan-2-ol (700 ml.), water (300 ml.) with NH_3 in vapour phase; (3) butan-1-ol (770 ml.), water (130 ml.), 98% formic acid (100 ml.); (4) butan-1-ol (860 ml.), water (140 ml.) with NH_3 in vapour phase.

Solvent	R_F 1	2	3	4
Guanine	0·26	0·49	0·09	0·08
1-Methylguanine	0·26	0·62	0·19	0·13
6-Hydroxy-2-methylaminopurine	0·50	0·66	0·25	0·18
2-Dimethylamino-6-hydroxypurine	0·43	0·69	0·28	0·20
2-Ethylamino-6-hydroxypurine	0·61	0·82	—	—
Guanosine	0·37	0·42	0·10	0·02
1-Methylguanosine	0·45	0·64	0·16	0·11
6-Hydroxy-2-methylaminopurine riboside	0·55	0·63	0·18	0·08
2-Dimethylamino-6-hydroxypurine riboside	0·49	0·66	0·23	0·10
Uracil	0·69	0·72	0·38	0·20
Uridine	0·70	0·70	0·21	0·08

Table 3. *Electrophoretic mobilities of the methylated guanosines*

Buffer	Mobility (cm./hr.) towards the anode at 20 v/cm. 0·05 M-borate (pH 9·2)	0·05 M-glycine (pH 10·0)	0·05 M-phosphate (pH 2·2)
Guanosine	+7·0	—	—
1-Methylguanosine	+5·0	−0·4	—
6-Hydroxy-2-methylaminopurine riboside	+5·9	+0·6	−4·5
2-Dimethylamino-6-hydroxypurine riboside	+5·9	—	−5·1
Cytidine	+5·4	—	−8·6

slowly migrating substance was identified as 1-methylguanosine and the faster-moving spot as 6-hydroxy-2-methylaminopurine riboside. Separation of the two nucleosides could also be obtained by chromatography in solvent 4 if the ammonia vapour concentration in the tank was sufficiently high. 1-Methylguanosine had the smaller R_F (Table 2). The following criteria were used in the identification of the two nucleosides. (i) Both had chromatographic properties in solvent systems 1–4 and electrophoretic mobilities in 0·05 M-borate, pH 9·2, and 0·05 M-glycine buffer, pH 10·0, identical with those of the corresponding enzymically synthesized nucleosides. Both nucleosides form borate complexes and in borate, pH 9·2, migrate towards the anode, but 6-hydroxy-2-methylaminopurine riboside has an additional charge due to the ionization of the 6-hydroxy group. This dissociation is absent in 1-methylguanosine since the hydrogen atom on $N_{(1)}$ is replaced by a methyl group, and the nucleoside migrates more slowly than the monomethylamino- and dimethylamino-guanosine derivatives. In glycine at pH 10·0, where the only charges are those due to the 6-hydroxy group, 1-methylguanosine remains stationary and the 6-hydroxy-2-methylaminopurine riboside migrates towards the anode (Table 3).

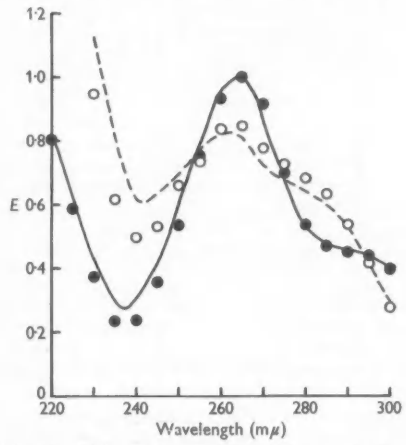


Fig. 1. Ultraviolet-absorption spectra of enzymically prepared 2-dimethylamino-6-hydroxypurine riboside in 0·1 N-HCl (——) and in 0·1 N-KOH (- - -). Points are for a sample isolated from the RNA of wheat embryo in 0·1 N-HCl (●) and in 0·1 N-KOH (○). For each sample the spectra in acid and alkali are for solutions of equal concentration, the spectrum in acid being plotted to give *E* 1·0 at 265 mμ.

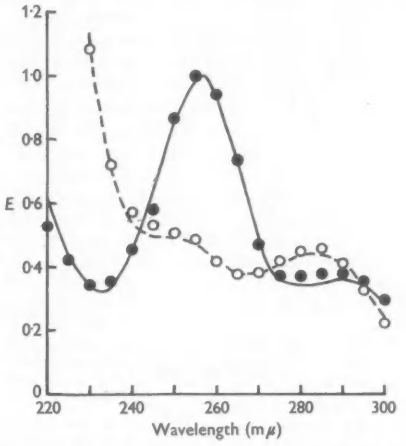


Fig. 2. Ultraviolet-absorption spectra of synthetically prepared 2-dimethylamino-6-hydroxypurine in 0·1 N-HCl (——) and in 0·1 N-KOH (- - -). Points are for a sample isolated from the RNA of wheat embryo in 0·1 N-HCl (●) and in 0·1 N-KOH (○). For each sample the spectra in acid and alkali are for solutions of equal concentration, the spectrum in acid being plotted to give *E* 1·0 at 256 mμ.

Table 4. *Principal ultraviolet-absorption maxima of the methylated guanines and their ribosides*

Inflexion points and subsidiary peaks are not given.

	$\lambda_{max.}$ (mμ)			
	Base		Riboside	
pH	1·0	13·0	1·0	13·0
Guanine*	248·5	274	256·5	258–266
1-Methylguanine	250	277	258	256
6-Hydroxy-2-methylaminopurine	251	278	258	258
2-Dimethylamino-6-hydroxypurine	256	283	265	262
2-Ethylamino-6-hydroxypurine†	253	—	—	—

* Data from Beaven, Holiday & Johnson (1955).

† Data from Elion, Lange & Hitchings (1956).

(ii) Comparison of the ultraviolet-absorption spectra of the natural and reference nucleosides at pH 1·0 and pH 13·0 (Figs. 3, 4; Table 4).

(iii) On hydrolysis in N-HCl the bases liberated from the nucleosides were identified as 1-methylguanine and 6-hydroxy-2-methylaminopurine respectively by comparison of their chromatographic properties in solvents 1–4 (Table 2) and their ultraviolet-absorption spectra (Figs. 5, 6; Table 4) with those of the synthetic purines.

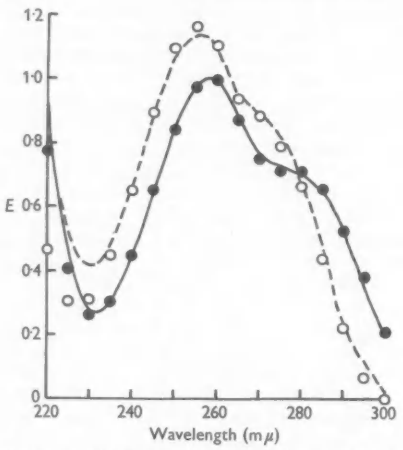


Fig. 3. Ultraviolet-absorption spectra of enzymically prepared 1-methylguanosine in 0·1 N-HCl (——) and in 0·1 N-KOH (- - -). Points are for a sample isolated from the RNA of *A. aerogenes* in 0·1 N-HCl (●) and in 0·1 N-KOH (○). For each sample the spectra in acid and alkali are for solutions of equal concentration, the spectrum in acid being plotted to give *E* 1·0 at 258 mμ.

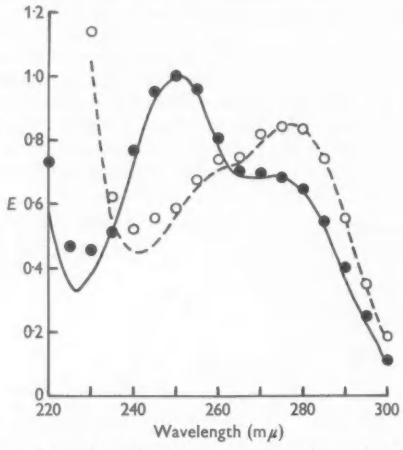


Fig. 5. Ultraviolet-absorption spectra of synthetically prepared 1-methylguanine in 0·1 N-HCl (——) and in 0·1 N-KOH (- - -). Points are for a sample isolated from the soluble fraction of rat-liver RNA in 0·1 N-HCl (●) and 0·1 N-KOH (○). For each sample the spectra in acid and alkali are for solutions of equal concentration, the spectrum in acid being plotted to give *E* 1·0 at 250 mμ.

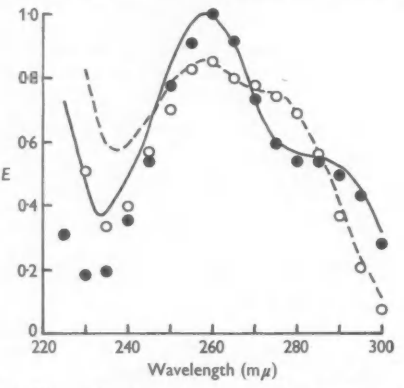


Fig. 4. Ultraviolet-absorption spectra of enzymically prepared 6-hydroxy-2-methylaminopurine riboside in 0·1 N-HCl (——) and in 0·1 N-KOH (- - -). Points are for a sample isolated from the soluble fraction of rat-liver RNA in 0·1 N-HCl (●) and in 0·1 N-KOH (○). For each sample the spectra in acid and alkali are for solutions of equal concentration, the spectrum in acid being plotted to give *E* 1·0 at 258 mμ.

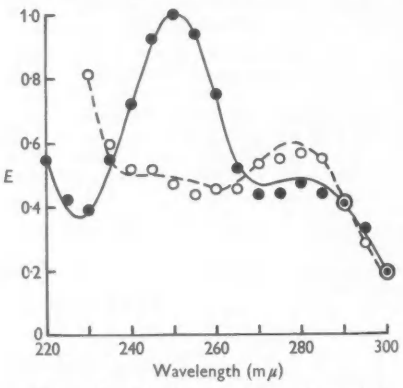


Fig. 6. Ultraviolet-absorption spectra of synthetically prepared 6-hydroxy-2-methylaminopurine in 0·1 N-HCl (——) and in 0·1 N-KOH (- - -). Points are for a sample isolated from the soluble fraction of rat-liver RNA in 0·1 N-HCl (●) and in 0·1 N-KOH (○). For each sample the spectra in acid and alkali are for solutions of equal concentration, the spectrum in acid being plotted to give *E* 1·0 at 251 mμ.

Stability of 1-methylguanosine. Dr P. D. Lawley (personal communication) has recently suggested that 1-methyladenylic acid is unstable at alkaline pH and undergoes a re-arrangement to yield 6-methylaminopurine ribonucleotide. Although no such interconversions have been described in guanine derivatives, we investigated the stability of 1-methylguanosine in alkali to eliminate the possibility that the nucleotide of 6-hydroxy-2-methylaminopurine had been derived from 1-methylguanylic acid by a similar methyl migration during alkaline hydrolysis of the RNA. A solution of 1-methylguanosine in 0·1 N-KOH was incubated at 37° for 18 hr. After neutralization with perchloric acid it was chromatographed in solvent 2 and then placed on electrophoresis in borate, pH 9·2, where the two nucleosides separate. No 6-hydroxy-2-methylaminopurine riboside was detected. Likewise the nucleoside of 6-hydroxy-2-methylaminopurine was unchanged by treatment in 0·1 N-KOH.

Although the sugar has not yet been isolated from any of the three nucleosides, their identity as ribosides is shown by the comparison of their chromatographic and electrophoretic properties with enzymically synthesized ribosides. Particularly, their electrophoretic mobilities in borate buffer, pH 9·2, were those expected from the formation of borate complexes on the 2′- and 3′-*cis*-hydroxyl groups. In the nucleosides of 1-methylguanine and 6-hydroxy-2-methylaminopurine, comparison of their electrophoretic mobilities in borate, pH 9·2, and glycine buffer, pH 10·0, more directly demonstrated borate-complex formation.

Separation of nucleoside 2′- and 3′-phosphates

From the mechanism of alkaline hydrolysis of RNA involving phosphoryl migration (Brown & Todd, 1952) the mononucleotides so produced should be a mixture of the nucleoside 2′- and 3′-phosphates. Paper chromatography in solvent 5 resolves the 2′- and 3′-phosphates of adenosine and guanosine. In this solvent the nucleotide of 2-dimethylamino-6-hydroxypurine, isolated from alkaline hydrolysates of wheat-embryo RNA, also separated into two substances migrating just behind guanosine 2′- and 3′-phosphates respectively. These had ultraviolet-absorption spectra at pH 1·0 which were both similar to those of the nucleoside.

Isolation of nucleosides from snake-venom digests of ribonucleic acid

The isolation of 1-methylguanosine and 2-dimethylamino-6-hydroxypurine riboside after hydrolysis of wheat-embryo RNA with the phosphodiesterase and 5′-nucleotidase present in crude snake venom provided confirmation that these nucleosides were bound by 3′:5′-phosphodiester linkages in the polynucleotide chain. Wheat-embryo RNA (50 mg.) was incubated with Russell viper venom at pH 9·0. The hydrolysate was chromatographed in solvent 2, where it separated into two major bands, one containing guanosine and a faster band which contained adenosine, cytidine and uridine. The latter material was eluted and separated by electrophoresis in 0·05 M-phosphate buffer, pH 2·2. Uridine, being uncharged, remained at the starting position whereas adenosine and cytidine migrated towards the negative electrode. A third band migrated behind adenosine with a mobility 1·1 times that of guanosine. This was re-chromatographed in solvent 2 to remove phosphate. The single ultraviolet-absorbing band was transferred to a second chromatogram and run in solvent 3, where it separated into two substances, one moving with a marker of 1-methylguanosine and a faster-moving substance migrating with 2-dimethylamino-6-hydroxypurine riboside. The identities of these were confirmed by their electrophoretic mobilities in borate buffer, pH 9·2, and their ultraviolet spectra at pH 1·0 and pH 13·0. On electrophoresis in borate the slower-moving substance in solvent 3 gave, in addition to 1-methylguanosine, a small spot with the mobility of 6-hydroxy-2-methylaminopurine riboside. There was insufficient of this material for its identification.

Distribution of methylated guanines in ribonucleic acids

RNA preparations from a variety of sources were examined for the presence of the three methylated guanines. As these compounds, like guanine, are very insoluble in water and tend to trail in most chromatographic systems it was easier to separate and estimate them as the more soluble ribosides. The nucleotides were isolated from alkaline hydrolysates as described above, dephosphorylated with prostate phosphomonoesterase, and the nucleosides isolated by chromatography in solvent 3 followed by electrophoresis in borate buffer, pH 9·2. They were identified by the criteria already described and estimated spectrophotometrically. The amounts were determined relative to uridine. The uridylic acid separated on the paper electrophoresis at pH 2·1 was re-chromatographed in solvent 2. It was then converted into uridine with prostate phosphomonoesterase, chromatographed in solvent 3 and estimated spectrophotometrically. The results, which are necessarily very approximate, are given in Table 5.

DISCUSSION

1-Methylguanine and 6-hydroxy-2-methylaminopurine have been found in the urine of normal human subjects, together with three other

Table 5. *Approximate molar proportions of methylated guanines in ribonucleic acids from several sources*

— Indicates less than 0·01 %.

Source of ribonucleic acid	1-Methylguanine	6-Hydroxy-2-methylaminopurine (moles/100 moles of uracil)	2-Dimethylamino-6-hydroxypurine
Aerobacter aerogenes	0·1	0·05	—
Yeast†	0·02	0·02	*
Wheat embryo	0·6	0·2	0·5
Nicotiana glutinosa leaves	0·2	*	0·06
Sugar-beet leaves	0·2	*	0·1
Rat-liver microsomes	0·09	0·08	0·09
Rat-liver soluble ribonucleic acid fraction	4·0	2·2	2·9
Turnip-yellow-mosaic virus	—	—	—
Tobacco-mosaic virus	—	—	*

* Not examined.

† Data of Adler, Weissmann & Gutman (1958).

methylated purines, 1-methylhypoxanthine, 7-methylguanine and 8-hydroxy-7-methylguanine (Weissmann, Bromberg & Gutman, 1957). 2-Dimethylamino-6-hydroxypurine, however, has not previously been reported to occur naturally. 1-Methylguanine and 6-hydroxy-2-methylaminopurine have been isolated as the bases from acid hydrolysates of yeast RNA by Adler *et al.* (1958). The isolation of the ribonucleosides and nucleotides from RNA described in this paper provides more conclusive evidence that these two purines and 2-dimethylamino-6-hydroxypurine are present in the polynucleotide chain as nucleotide residues bound by 3′- and 5′-phosphodiester linkages.

The distribution of these purines in RNA preparations from different biological sources shows many similarities with that of the methylated adenines (Littlefield & Dunn, 1958*a*, *b*). The methylated guanines were not detected in the ribonucleic acids of turnip-yellow-mosaic and tobacco-mosaic viruses. If present they would represent less than one residue per virus particle. Of other RNA preparations examined only in that from *A. aerogenes* was one of the three methylated guanines (2-dimethylamino-6-hydroxypurine) definitely shown to be absent.

The soluble RNA fraction from rat liver differed from the other nucleic acids in having unusually high proportions of the methylated guanines and also of 6-methylaminopurine and the additional pyrimidine nucleotide described by Cohn and Davis & Allen (Dunn & Smith, 1959*a*). The RNA from plant tissues and micro-organisms was prepared by methods which isolate almost all the RNA in the cell (in the isolation of wheat-embryo RNA the nuclear RNA is possibly discarded). It is thus quite possible that the methylated purines found in these preparations may be largely or exclusively present in a low-molecular-weight RNA similar to the rat-liver soluble RNA.

SUMMARY

1. 6-Hydroxy-2-methylaminopurine, 2-dimethylamino-6-hydroxypurine and 1-methylguanine have been detected in ribonucleic acid from several sources in amounts varying from 0·05 to 4 % of the uracil.

2. The compounds were isolated as the ribosides and identified by chromatographic and spectroscopic comparison with synthetic samples. Each of the ribosides was converted into the base, which was similarly identified. The ribonucleoside 2′- and 3′-phosphates have also been isolated, and other evidence indicates that the three nucleosides were originally present in 3′:5′-phosphodiester linkage in the ribonucleic acid.

3. None of the methylated guanines was found in the ribonucleic acids of tobacco-mosaic and turnip-yellow-mosaic viruses. 2-Dimethylamino-6-hydroxypurine was absent from the ribonucleic acid of *Aerobacter aerogenes*. The highest proportions of the methylated guanines were found in the soluble ribonucleic acid fraction of rat liver.

We wish to express our thanks to Dr G. H. Hitchings and Professor D. Keilin, F.R.S., for gifts of chemicals, and to Dr A. R. Trim and Dr M. B. Hoagland for samples of ribonucleic acids. We also wish to thank Dr Trim for giving details of his method for isolation of plant RNA before this was published.

REFERENCES

Adler, M., Weissmann, B. & Gutman, A. B. (1958). *J. biol. Chem.* **230**, 717.

Amos, H. & Korn, M. (1958). *Biochim. biophys. Acta*, **29**, 444.

Beaven, G. H., Holiday, E. R. & Johnson, E. A. (1955). In *The Nucleic Acids*, vol. 1. Ed. by Chargaff, E. & Davidson, J. N. New York: Academic Press Inc.

Brown, D. M. & Todd, A. R. (1952). *J. chem. Soc.* p. 52.

Cohn, W. E. (1957). *Fed. Proc.* **16**, 166.

Cohn, W. E. (1958). *Fed. Proc.* **17**, 203.

Cohn, W. E. (1959). *Biochim. biophys. Acta*, **32**, 569.
Davis, F. F. & Allen, F. W. (1957). *J. biol. Chem.* **227**, 907.
Dunn, D. B. & Smith, J. D. (1959*a*). *Trans. Faraday Soc.* (in the Press).
Dunn, D. B. & Smith, J. D. (1959*b*). *Proc. 4th Int. Congr. Biochem.* (in the Press).
Elion, G. B., Lange, W. H. & Hitchings, G. H. (1956). *J. Amer. chem. Soc.* **78**, 217.
Ginoza, W., Atkinson, D. E. & Wildman, S. G. (1954). *Science*, **119**, 269.
Hoagland, M. B., Stephenson, M. L., Scott, S. F., Hecht, L. I. & Zamecnik, P. C. (1958). *J. biol. Chem.* **231**, 241.
Littlefield, J. W. & Dunn, D. B. (1958*a*). *Nature, Lond.*, **181**, 254.
Littlefield, J. W. & Dunn, D. B. (1958*b*). *Biochem. J.* **70**, 642.
Markham, R. (1955). In *Modern Methods of Plant Analysis*, vol. 4. Ed. by Paech, K. & Tracey, M. V. Berlin: Springer Verlag.
Markham, R. & Smith, J. D. (1949). *Biochem. J.* **45**, 294.
Markham, R. & Smith, J. D. (1950). *Biochem. J.* **46**, 513.
Markham, R. & Smith, J. D. (1951). *Biochem. J.* **49**, 401.
Markham, R. & Smith, J. D. (1952). *Biochem. J.* **52**, 552.
Markham, R. & Smith, K. M. (1949). *Parasitology*, **39**, 330.
Paege, L. M. & Schlenk, F. (1952). *Arch. Biochem. Biophys.* **40**, 42.
Smith, J. D. & Markham, R. (1950). *Biochem. J.* **46**, 509.
Weissmann, B., Bromberg, P. A. & Gutman, A. B. (1957). *J. biol. Chem.* **224**, 407.
Wyatt, G. R. (1951). *Biochem. J.* **48**, 584.

Degradation of the Hyaluronic acid Complex of Synovial Fluid by Proteolytic Enzymes and by Ethylenediaminetetra-acetic acid

By A. G. OGSTON and T. F. SHERMAN
Department of Biochemistry, University of Oxford

(*Received* 3 *December* 1958)

Blumberg & Ogston (1957*a*, *b*) observed that papain in the presence of ethylenediaminetetra-acetic acid hydrolysed the protein present in the hyaluronic acid complex of ox-synovial fluid, strongly reduced the non-Newtonian viscosity of the complex and caused a considerable drop in its molecular weight. They concluded that the protein of this complex is essential to the integrity of its macromolecular structure and to its physicochemical properties in solution and, consequently, that the effect of papain on the viscosity of hyaluronic acid from other sources may provide evidence of its existence as a complex with protein.

Experiments done in collaboration with Dr B. I. Aldrich (Aldrich, 1958) in which ethylenediaminetetra-acetic acid was used in an attempt to remove calcium from the complex, showed that buffered solutions of complex containing ethylenediaminetetra-acetic acid suffered loss of viscosity during measurements in the stainless-steel Couette viscometer, but that this did not occur when an analogue of ethylenediaminetetra-acetic acid was used instead, nor when ethylenediaminetetra-acetic acid was used in a glass viscometer. These results suggested that ethylenediaminetetra-acetic acid might be concerned in catalytic degradation of the carbohydrate component of the complex; they thus also cast doubts upon the conclusions of Blumberg & Ogston, whose work with papain involved the use of ethylenediaminetetra-acetic acid as an activator and of the stainless-steel viscometer. Further investigations on the effects of ethylenediaminetetra-acetic acid and of proteolytic enzymes on the complex were therefore undertaken.

EXPERIMENTAL

Materials

Hyaluronic acid complex. (Complex.) Synovial fluid was collected from the astragalotibial joints of oxen soon after death, as described by Ogston & Stanier (1950). The fluid was centrifuged in a Spinco model L ultracentrifuge at 73 000 *g* for 60 min. to remove particulate matter, and the complex was separated by filtration, with 9–11 washings, at 4° on a 5-on-3 sintered-glass filter of 1 μ (or less) average pore diameter (Baird and Tatlock Ltd.). The buffer used for washing was 0·2 M-NaCl–0·0077 M-Na_2HPO_4–0·0023 M-KH_2PO_4 (pH 7·3). The complex used for experiments with trypsin, chymotrypsin and pepsin was finally dissolved in this same buffer to approximately the volume of the original fluid; that used for experiments with papain and cysteine was dissolved in 0·0453 M-Na_2HPO_4–0·0213 M-KH_2PO_4 buffer (pH 7·0) to about half the volume of the original fluid. In all cases, the complex was then centrifuged at 73 000 *g* for 45 min. and dialysed against the buffer in which it was finally dissolved. Complex that was used for the determination of proteolysis by the method of Anson (1938) was further centrifuged at 105 000 *g* for 180 min. to remove any 'fast component' (Blumberg & Ogston, 1957*a*; Johnston, 1955).

Trypsin. This was Armour salt-free material of stated activity 1800 units/mg.

Chymotrypsin. This was Armour salt-free material of stated activity 975 units/mg.

Pepsin. This was Armour salt-free material.

Papain. This was twice-crystallized from crude papain (British Drug Houses Ltd.) by the method of Kimmel & Smith (1954). Stock solutions were made by dissolving freeze-dried material to 1 g./100 ml. in 0·0453 M-Na_2HPO_4–0·0213 M-KH_2PO_4 buffer (pH 7·0). In some of the experiments a sample of papain obtained from Schering A. G., Berlin, and of about the same activity as that mentioned above, was used in the same manner. We are also indebted to Mr C. F. Phelps for a sample also prepared by the method of Kimmel & Smith.

Ox-serum albumin. This was from material isolated by Dr B. S. Blumberg (Blumberg & Ogston, 1957*a*).

Ethylenediaminetetra-acetic acid. (Hereafter denoted by EDTA.) The same sample was used as that used by Blumberg & Ogston (1957*a*).

Ethylene glycol bis-(β-aminoethyl ether)-NN-tetra-acetic acid. (Hereafter denoted by EGTA.) This was kindly supplied by Dr W. Bartley from a sample obtained from J. R. Geigy, S.A., Basle, Switzerland.

Cysteine. This was British Drug Houses Ltd. laboratory-grade reagent.

Metal salts. These were from British Drug Houses Ltd., analytical-grade reagents or equivalent.

Methods

Viscosity. This was measured with the Couette viscometer of Ogston & Stanier (1953) at 25°, with velocity gradients approximately between 0·4 and 40·0 sec.$^{-1}$. Viscosity at high-velocity gradient was measured with an Ostwald viscometer of about 2·5 ml. capacity and water time 48 sec.

Electrodialysis. This was performed with an apparatus designed and constructed by Dr O. Smithies (Smithies, 1951) and previously described (Ogston & Sherman, 1958). Samples were placed in the central compartment, which was separated from the outer chambers by Millipore (Millipore Filter Corp., Watertown, Mass., U.S.A.) HA membranes of pore diameter 0·4 μ, and electrodialysed for 9–11 hr. Buffer, 0·0153 M-KH_2PO_4–0·0097 M-Na_2HPO_4 (pH 6·7), was circulated through the outer chambers. The entire apparatus was used in the cold room at about 2°.

Ultraviolet absorption. This was measured at 280 mμ (corrected for scatter at 350 mμ) in a Hilger Uvispek spectrophotometer.

Mucin clot. (*a*) Quantitative: Mucin weights were determined by heating the clot formed by acidification of 1 ml. samples of hyaluronic acid solutions (Ogston & Stanier, 1952) to constant weight at 105° in an electric oven. Hyaluronic acid complex was taken as 45% of the mucin weight (Ogston & Stanier, 1952).

(*b*) Qualitative: The type of clot formed by acidification of samples of hyaluronic acid was noted, as the action of some agents upon hyaluronic acid causes a dispersed precipitate to be formed instead of the normal compact clot (Blumberg, 1957).

Treatment of the complex with trypsin and chymotrypsin. Weighed amounts of trypsin and chymotrypsin were added directly to buffered solutions (0·2 M-NaCl–0·0077 M-Na_2HPO_4–0·0023 M-KH_2PO_4, pH 7·3) of the complex to the extent of 0·5 mg. of each per 1 ml. of solution. Incubations were at 4°.

Treatment of the complex with pepsin. The complex was first dialysed against 0·18 M-NaCl–0·01 M-HCl (pH 2·0). Pepsin was then added directly to the resulting solution of the complex to the extent of 1 mg. per 1 ml. of solution. Incubations were at 4°.

Treatment of the complex with papain. Stock (1%) solution of papain was first activated by addition of KCN and EDTA in amounts to give concentrations of 5 mM in the reaction mixture, or for EGTA to give a concentration of 2·5 mM in the reaction mixture. (In one experiment EDTA was also used at 2·5 mM.) The reaction mixture was made by adding 1 ml. of the activated papain solution, adjusted to pH 7 by the addition of a very small quantity of 0·2 M-HCl, to 9 ml. of a solution of the complex (pH 7·0). Incubations were at 4°.

Treatment of the complex with cysteine. This was carried out in the same manner as for papain, except that the stock cysteine solution was made to give a reaction mixture of 0·4 mM-cysteine (equivalent to about the same SH-group concentration as the papain reaction mixture). KCN and EDTA or EGTA were added as with the papain.

Activity of papain. This was determined by the Greenberg (1955) modification of the method of Anson (1938) with ox-serum albumin as substrate.

Detection of proteolysis of the complex. (*a*) With papain: This was accomplished by the Blumberg (1957) modification of the method of Anson (1938), except that ox-serum albumin was added to the digestion mixture immediately before the addition of trichloroacetic acid (but after the solution had been cooled to 4° and acidified to pH 5·0), to assist the precipitation of hyaluronic acid; the albumin [3 parts to 1 (w/w) of hyaluronic acid] was added in 2 ml. of water. Control experiments were done to measure any self-digestion of the papain.

(*b*) With trypsin and chymotrypsin, and with pepsin: Protein content (measured by ultraviolet absorption at 280 mμ) of a sample of complex incubated with the enzyme and then isolated by electrodialysis was compared with that of an undigested control; allowance was made for a small loss of hyaluronic acid during electrodialysis, as determined by mucin clot weights. A lower protein content in this material indicated that proteolysis had occurred, as electrodialysis itself does not remove any protein from the complex (Ogston & Sherman, 1958).

RESULTS

Effects of trypsin and chymotrypsin and of pepsin on the complex. Treatment of the complex with trypsin and chymotrypsin together for 7 days had no detectable effect upon the viscosity, which was compared in the Couette viscometer with a control sample incubated without enzymes (Fig. 1). The mucin clots obtained from the digested and control samples were identical both in appearance and in weight/ml. of solution. The occurrence of proteolysis was demonstrated in a sample digested for 5 days and then electrodialysed, by comparing the absorption at 280 mμ with the weight of mucin clot in this and in a control sample; this showed that 65% of the protein originally present had been removed. In the 5-days' experiment, 14% of the polysaccharide was lost during electrodialysis, so that the viscosity after treatment was not

directly comparable with that of the control; however, the ratio of viscosities at velocity gradients 0 and 50 sec.$^{-1}$ (Ogston & Stanier, 1953) was normal, showing that its viscous properties were unaffected.

Preliminary experiments only were done with pepsin; these indicated that very little protein could be eliminated with this enzyme, and its use was therefore abandoned. The observation was made, however, that the complex could be kept at pH 2·0 for 2 days without any change of non-Newtonian viscosity.

Effects of ethylenediaminetetra-acetic acid and of EGTA on the complex. EDTA alone caused a complete disappearance of non-Newtonian viscosity and a lowering of viscosity at all velocity gradients within 1 hr. at 25° in the Couette viscometer. Material which had been so treated showed a marked reduction of viscosity as compared with a control sample kept in glass, when tested in the glass Ostwald viscometer. A solution of complex in EDTA showed no loss of viscosity in the Ostwald viscometer, even when sheared by repeated passage through the capillary. When EDTA was replaced by EGTA very little change of viscosity occurred, even in the Couette viscometer for 1 hr. at 25°.

The apparent implication of the material (stainless steel) of the Couette viscometer in this effect led to the testing in the glass Ostwald viscometer of the effects of a number of ions (Fe^{3+}, Cr^{3+}, Ni^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}) added in a final concentration of about 20 mM to synovial fluid and EDTA. None of these ions, all in their highest stable states of oxidation, had any significant effect upon the viscosity. However, Sn^{2+}, Cu^{+} and Fe^{2+} ions caused loss of viscosity; with Fe^{2+} ions the degradation was not dependent upon the presence of EDTA, and appeared to be inhibited at low concentrations of Fe^{2+} ions by EGTA. When ascorbic acid (25 mM) was added to the complex, the viscosity (in glass) was reduced; this effect was increased by inclusion of EDTA but inhibited by EGTA.

Effects of papain and of cysteine on the complex. The activity of papain upon ox-serum albumin was found to be identical in the presence of EDTA and of EGTA. It was confirmed that papain causes proteolysis of the complex in the presence of 5 mM-EDTA (Blumberg & Ogston, 1957*a*); identical results were obtained in the presence of 2·5 mM-EGTA.

However, the effects of papain on the non-Newtonian viscosity were much greater with EDTA

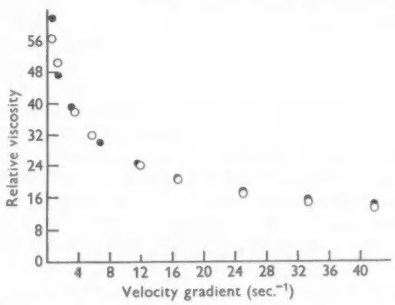


Fig. 1. Variation of relative viscosity with velocity gradient, measured in the Couette viscometer in buffer, pH 7·3, at 25°. ●, Hyaluronic acid complex treated with trypsin and chymotrypsin for 7 days at 4°; ○, untreated complex.

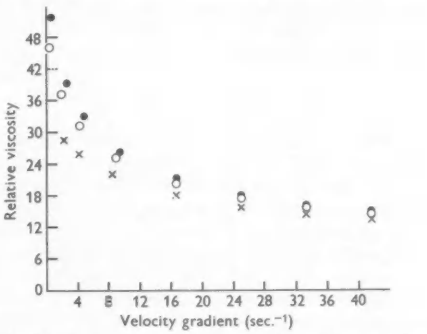


Fig. 2. Variation of relative viscosity with velocity gradient, measured in the Couette viscometer in buffer, pH 7·0, at 25°. ●, Control: hyaluronic acid complex treated only with EGTA and KCN; ○, complex treated with papain, EGTA and KCN for 24 hr. at 4°; ×, complex treated with papain, EDTA and KCN for 24 hr. at 4°.

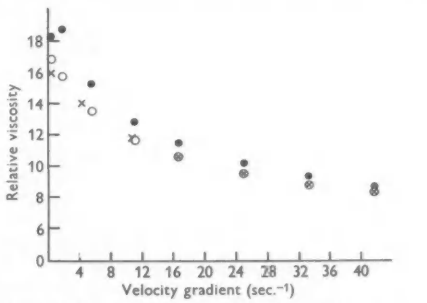


Fig. 3. Variation of relative viscosity with velocity gradient, measured in the Couette viscometer in buffer, pH 7·0, at 25°. ●, Control: hyaluronic acid complex treated only with EDTA and KCN; ○, complex treated with papain, EDTA and KCN for 25 hr. at 4°; ×, complex treated with cysteine, EDTA and KCN for 25 hr. at 4°.

than with EGTA (Fig. 2). The change of viscosity with papain and EDTA was markedly greater than with EDTA alone. In view of the apparent unimportance of proteolysis, a careful comparison was made of the effects of papain and cysteine (at the same concentration of SH groups) with EDTA and EGTA measured in the Couette viscometer. With EDTA (Fig. 3) and with EGTA (Fig. 4) the effects of papain and cystein were found to be closely similar. The only difference that could be observed between the actions of cysteine and papain on viscosity was that addition of 0·1 mM-Cu^{2+} ions increased the effect of cysteine with EDTA, but decreased that of papain.

After incubation of the complex with cysteine and EDTA in glass, the mucin clot formed was abnormal, resembling that formed after similar treatment with papain.

DISCUSSION

Action of ethylenediaminetetra-acetic acid on the hyaluronic acid complex. Although we have not worked out the nature of the effect in detail, it seems clear that EDTA can assist in a non-proteolytic degradation of the complex, and it is likely that this is an oxidative process exerted upon the hyaluronic acid. The effect of EDTA on the viscosity, in the presence of ascorbic acid in glass, suggests that a complex between EDTA and a metal ion present in the preparation acts as an oxidation catalyst (Pirie & van Heyningen, 1954; Udenfriend, Clark, Axelrod & Brodie, 1954; Stadtman, 1955; Green, Mazur & Schorr, 1956; Mason, 1957). The effect of EDTA and cysteine (in glass) upon the ability to form a mucin clot suggests that cysteine also can act as a reducing agent in the system (Hale, 1944). The effect of EDTA, when observed in the stainless-steel viscometer, together with the effects (in glass) of EDTA and Sn^{2+} or Cu^{+} ions, suggests that EDTA may also act by bringing such ions into solution from the steel. EGTA appears to be far less active in the catalytic role than is EDTA, and even to act as an inhibitor of the reaction with ascorbic acid.

In view of this, the use of EDTA in preparing hyaluronic acid from tissues appears to be most undesirable, since both metal ions capable of forming an oxidation catalyst with EDTA and reducing agents (apart from papain) are likely to be present, and the product is therefore likely to have undergone oxidative degradation. Stadtman (1955) has issued a strong warning against its use to protect thiols against oxidation in enzyme systems.

Effects of proteolytic enzymes on the hyaluronic acid complex. We have confirmed Blumberg & Ogston (1957*a*) in showing that papain does hydrolyse the protein of the complex, but we have shown that trypsin and chymotrypsin together have a proteolytic action also (which Blumberg & Ogston did not find).

Since the actions of the non-SH enzymes produce no change of viscosity or of the ability to form mucin, the action of papain on these properties, which is closely imitated by cysteine, cannot now be ascribed to proteolysis. It is still possible that papain, under the conditions used, may affect the viscosity by a more complete proteolysis than that produced by trypsin and chymotrypsin, but this would not be easy to prove in the presence of the oxidative degradation. Blumberg & Ogston's (1957*b*) finding that plasmin causes proteolysis and loss of viscosity (in the absence of EDTA) is inconclusive because of the ill-defined nature of the enzyme.

It follows that any change of viscosity brought about by papain cannot be taken as evidence for the existence of a complex, as Blumberg & Ogston (1957*b*) claimed; conversely, lack of change of viscosity on proteolysis is not evidence against the existence of a complex.

Hyaluronic acid–protein complex of ox-synovial fluid. These results do nothing to contradict the earlier evidence for the existence of a characteristic complex between hyaluronic acid and protein in synovial fluid (Ogston & Stanier, 1950, 1952; Curtain, 1955), which has been confirmed by the more recent evidence of electrodialysis (Ogston & Sherman, 1958) and of the composition of material isolated by precipitation with cetylpyridinium chloride (Blumberg & Ogston, 1957*b*). They do strongly suggest, however, that this protein, or a

Fig. 4. Variation of relative viscosity with velocity gradient, measured in the Couette viscometer in buffer, pH 7·0, at 25°. ●, Control: hyaluronic acid complex treated only with EGTA and KCN; ○, complex treated with papain, EGTA and KCN for 48 hr. at 4°; ×, complex treated with cysteine, EGTA and KCN for 48 hr. at 4°.

considerable part of it, is not important in determining the physicochemical properties of hyaluronic acid in solution. Although it remains true that no sample free from protein appears to have been isolated with so high an intrinsic viscosity as that of the complex it seems likely that this is because methods powerful enough to remove all the protein can also cause depolymerization of the polysaccharide.

SUMMARY

1. Ethylenediaminetetra-acetic acid aids the degradation of hyaluronic acid complex under various conditions.

2. Papain has a proteolytic activity on the complex but appears, as a reducing agent, to cause more general degradation as well.

3. Trypsin and chymotrypsin together can remove 65% of the protein from the complex without any loss of viscosity by the complex.

We are grateful to Dr H. Gibian of Schering A.G. for a gift of papain, and to Dr B. S. Blumberg for information and advice. One of us (T.F.S.) is indebted to the Arthritis and Rheumatism Foundation, New York, for a grant under which a part of this work was done.

REFERENCES

Aldrich, B. I. (1958). D.Phil. Thesis: Oxford.
Anson, M. (1938). *J. gen. Physiol.* **22**, 79.
Blumberg, B. S. (1957). D.Phil. Thesis: Oxford.
Blumberg, B. S. & Ogston, A. G. (1957*a*). *Biochem. J.* **66**, 342.
Blumberg, B. S. & Ogston, A. G. (1957*b*). *Biochem. J.* **68**, 183.
Curtain, C. C. (1955). *Biochem. J.* **61**, 688.
Green, S., Mazur, A. & Schorr, E. (1956). *J. biol. Chem.* **220**, 237.
Greenberg, D. M. (1955). In *Methods in Enzymology*, vol. 2, p. 54. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Hale, C. W. (1944). *Biochem. J.* **38**, 362.
Johnston, J. P. (1955). *Biochem. J.* **59**, 626.
Kimmel, J. R. & Smith, E. L. (1954). *J. biol. Chem.* **207**, 515.
Mason, H. S. (1957). In *Advances in Enzymology*, vol. 19, p. 79. Ed. by Nord, F. F. New York: Interscience Publishers.
Ogston, A. G. & Sherman, T. F. (1958). *Nature, Lond.*, **181**, 482.
Ogston, A. G. & Stanier, J. E. (1950). *Biochem. J.* **46**, 364.
Ogston, A. G. & Stanier, J. E. (1952). *Biochem. J.* **52**, 149.
Ogston, A. G. & Stanier, J. E. (1953). *Biochem. J.* **53**, 4.
Pirie, A. & van Heyningen, R. (1954). *Nature, Lond.*, **173**, 873.
Smithies, O. (1951). D.Phil. Thesis: Oxford.
Stadtman, E. R. (1955). In *Amino Acid Metabolism*, p. 418. Ed. by McElroy, W. D. & Glass, B. Baltimore: Johns Hopkins Press.
Udenfriend, S., Clark, C. T., Axelrod, J. & Brodie, B. B. (1954). *J. biol. Chem.* **208**, 731.

Factors Influencing the Incorporation of Amino Acid into the Protein of Microsome and Mitochondria Preparations of Rat Liver and Liver Tumour

By OLGA GREENGARD and P. N. CAMPBELL
Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London, W. 1

(*Received* 28 *November* 1958)

The requirement of the soluble cytoplasm (cell sap) for the incorporation of amino acids into the protein of isolated liver microsomes has been demonstrated by Zamecnik & Keller (1954). Both for microsomes derived from rat liver and mouse Ehrlich ascites tumour cells, the active components of the cell sap are precipitated when the pH is adjusted to 5·2 (pH 5 fraction) (Keller & Zamecnik, 1956; Littlefield & Keller, 1957). It has previously been observed that microsome preparations from rat-liver tumours are considerably less active for amino acid incorporation than are similar preparations from normal liver (Campbell & Greengard, 1959). Further, it appeared that not only were the microsomes deficient in this respect, but that the cell sap contributed to this difference. The extent to which the pH 5 fraction from the two tissues reflects the differences found with the whole cell sap has now been investigated.

For amino acid incorporation into isolated mitochondria, the role of the cell sap seems to differ from that with microsomes. In the presence of a suitable source of energy, there is no absolute requirement for cell sap (McLean, Cohn, Brandt & Simpson, 1958; Greengard, 1959), although its inclusion in the reaction mixture enhances the incorporation of amino acids. Other factors influencing the extent of incorporation of amino

acids into mitochondrial protein have now been studied. It is shown that the properties of tumour mitochondria are qualitatively similar to those derived from normal liver.

MATERIALS AND METHODS

Chemicals. Adenosine triphosphate (dipotassium salt; ATP) and guanosine triphosphate (sodium salt; GTP) were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Solutions of these substances were brought to pH 7·3 (indicator papers) with KOH before use. The barium–silver salt of phosphoenolpyruvic acid was prepared by the method of Ohlmeyer (1951), and the barium salt of fructose 1:6-diphosphoric acid was obtained from C. F. Boehringer und Soehne, Mannheim, Germany. Solutions of the free acids were prepared shortly before use and adjusted to pH 7·3 (indicator papers) with KOH. Crystalline ribonuclease was obtained from Armour and Co. Ltd., Eastbourne, Sussex and from Worthington Biochemical Corp., New Jersey, U.S.A. Pyruvic kinase was that of C. F. Boehringer und Soehne, Mannheim.

Radioactive amino acids. Uniformly labelled L-[^{14}C]-leucine (100 μc = 1·49 mg.) and L-[^{14}C]lysine (100 μc = 1·13 mg.) were obtained from The Radiochemical Centre, Amersham, Bucks.

Tissues. (1) Normal liver. This was obtained from rats (150 g. body wt.) fed on stock diet. (2) Liver tumours. Tumours were induced in rats by feeding them on a diet containing 0·06% of 4-dimethylaminoazobenzene as described by Campbell (1955). All rats bearing tumours were kept on a normal diet free of the carcinogen for at least 4 weeks before use.

Tissue preparations. (1) Microsomes. The method used followed closely that described by Zamecnik & Keller (1954) and was exactly as described previously (Campbell & Greengard, 1959). (2) Mitochondria. The method was based on that of Schneider & Hogeboom (1950) and was the same as that previously described (Campbell & Greengard, 1959), except that the mitochondria were washed twice instead of once. (3) pH 5 fraction. The cell sap (105 000 *g* for 50 min. supernatant) was brought to pH 4·9–5·1 (glass electrode) by the addition of M-acetic acid. The precipitate was centrifuged down, washed with 0·25 M-sucrose and resuspended in a volume of medium equivalent to one-tenth of the original volume of cell sap. The pH 5 fraction prepared thus had a protein content of about 1·5% (w/v).

Method of incubation. Microsomes or mitochondria (7–10 mg. of protein in each tube) were incubated in a total volume of 1 ml. at 37° for 50 min. under a continuous stream of $N_2 + CO_2$ (95:5) or $O_2 + CO_2$ (95:5), with 0·5 μc of ^{14}C-labelled amino acid, together with a suitable substrate as explained in the legends to the Tables. When studying the incorporation into the pH 5 fraction the preparation was incubated for 10 min. in air, with or without ATP.

Isolation of protein from the particulate fractions. After incubation, the microsomes or mitochondria were separated from the soluble constituents by centrifuging. The proteins were precipitated in aqueous 5% (w/v) trichloroacetic acid and were then treated with hot trichloroacetic acid and organic solvents according to a modified Schneider procedure (Zamecnik, Loftfield, Stephenson & Steele, 1951).

Treatment of the pH 5 *fraction and estimation of ribonucleic acid.* The pH 5 fraction was treated after incubation with 5 vol. of 0·4 N-perchloric acid and the precipitate washed with cold perchloric acid and organic solvents as described by Hoagland, Stephenson, Scott, Hecht & Zamecnik (1958). After determination of the specific radioactivity of the washed and dried pH 5 fraction, the ribonucleic acid (RNA) was extracted by heating for 45 min. in 5% (w/v) trichloroacetic acid and its quantity estimated by the orcinol method of Mejbaum (1939). The protein residue from the trichloroacetic acid extraction was washed with organic solvents, dried and weighed and its specific radioactivity measured.

Measurement of radioactivity. The material was compressed on a 0·28 cm.^{2} stainless-steel disk with a stainless-steel pestle, as described by Busch, Simbonis, Anderson & Greene (1956), and counted at infinite thickness in a Tracerlab SC-16 windowless flow counter. The accuracy of counting was as described previously (Campbell & Greengard, 1959).

RESULTS

Incorporation of [^{14}C]*leucine into the microsomal protein of liver and liver tumour*

In the presence of a source of energy-rich phosphate, microsomes of liver and liver tumour incorporate amino acid when the pH 5 fraction of either of these tissues is present in the incubation mixture. In Table 1 the effectiveness of the pH 5 fraction derived from the two hepatic tissues is compared. It can be seen that the results are not influenced by increasing the quantity of pH 5 fraction present. The highest extent of incorporation of leucine occurs when liver microsomes are incubated with liver pH 5 fraction. Neither the

Table 1. *Incorporation of* [^{14}C]*leucine into microsomal protein of liver and liver-tumour preparations*

Microsomes and pH 5 fraction (in quantities given under Methods) were incubated under $N_2 + CO_2$ (95:5) for 50 min. with 2 μmoles of ATP, 10 μmoles of phosphoenolpyruvate (PEP), PEP-kinase, 0·25 μmole of GTP and 0·5 μc of [^{14}C]-leucine. The microsomes were collected by centrifuging after incubation. Radioactivity is expressed as counts/min. at infinite thickness/0·28 cm.^{2} in a windowless flow counter. Control values, obtained by incubating the same preparations in the absence of PEP and ATP, were less than 10% of the experimental results.

	Liver microsomes				Tumour microsomes			
Origin of pH 5 fraction	Liver		Tumour		Liver		Tumour	
Amount of pH 5 fraction (ml.)	0·3	0·6	0·3	0·6	0·3	0·6	0·3	0·6
Radioactivity of microsomal protein	432	428	74	80	86	76	98	99

Table 2. *Incorporation of amino acid into the* pH 5 *ribonucleic acid of liver and liver tumour*

The pH 5 fraction (0·7 ml.) was incubated in air for 10 min. at 37° and was then treated with perchloric acid and organic solvents as described under Methods. Radioactivity is expressed as in Table 1.

	Radioactivity			
	Liver pH 5 fraction		Liver-tumour pH 5 fraction	
Amino acid	Control	Plus ATP (5 μmoles)	Control	Plus ATP (5 μmoles)
[^{14}C]Leucine	2	719	10	460
[^{14}C]Lysine	38	344	63	132

Table 3. *Incorporation of* [^{14}C]*leucine into and the ribonucleic acid/protein content of* pH 5 *fractions from liver and liver tumour*

The pH 5 fractions were incubated in air for 10 min. at 37° with 5 μmoles of ATP, then treated with perchloric acid (PCA). After treatment of the precipitate with organic solvents the radioactivity was determined and is expressed as in Table 1. The RNA was extracted from the precipitate with trichloroacetic acid and the amount estimated by the orcinol method of Mejbaum (1939). The radioactivity of the remaining protein was less than 10% of that before extraction.

Origin of pH 5 fraction	Expt. no.	Radioactivity	Amount of material incubated [PCA ppt. (mg.)]	$\frac{\text{RNA}}{\text{Protein}} \times 100$
Liver	1	691	11·7	5·4
	2	976	10·7	6·5
Liver tumour	1	284	9·4	6·5
	2	408	11·3	6·4

combination of liver pH 5 fraction with tumour microsomes nor that of liver microsomes with tumour pH 5 fraction raises the extent of incorporation above that obtained with tumour constituents alone.

Incorporation of amino acid into the pH 5 *ribonucleic acid of liver and liver tumour*

It has been demonstrated that amino acids are incorporated into the RNA moiety of the pH 5 fraction (pH 5 RNA) before their transfer to microsomal protein (Hoagland *et al.* 1958). It was therefore of interest to determine the extent of incorporation of amino acids into the pH 5 RNA derived from the two different tissues. The results shown in Table 2 indicate that, in the presence of an ATP-generating system, incorporation into the extract from the tumour tissue does occur, although the extent of this incorporation is lower than that for the extract of normal tissue. This is so both for [^{14}C]leucine and [^{14}C]lysine. Since treatment of the perchloric acid precipitate with hot trichloroacetic acid in all cases reduced the radioactivity of the precipitates to that of the control, lacking ATP, it is concluded that the incorporation was associated with the pH 5 RNA. These results are in accord with those of Hoagland *et al.* (1958).

Provided that the ratio of the amount of material incubated (i.e. weight of perchloric acid precipitate) to the amount of radioactive leucine was similar, two reasons for the lower extent of labelling of the tumour pH 5 fraction compared with that of normal liver may be considered: (i) a difference in the proportion of RNA to protein in the extracts; (ii) a lower catalytic activity of the extract. A further series of experiments were carried out in which the incorporation of [^{14}C]-leucine was studied and the RNA content of each extract determined. The RNA/protein ratio of the fractions prepared from different tumours showed considerable variation, but in the examples given in Table 3 it will be seen that the fractions of the two tissues were of similar composition. In spite of this a marked difference in the extent of the incorporation into the pH 5 RNA was found.

Incorporation of amino acid by mitochondria from liver and liver tumour

The mitochondria used in these studies were prepared in such a way as to minimize contamination by microsomes. In order to obtain a measure of such contamination the RNA content was estimated, and was found to be 1·5–2·0 mg./100 mg. of protein both in liver and liver-tumour mitochondria.

In the presence of a suitable ATP-generating system mitochondria from both liver and liver tumour incorporated amino acid into their proteins, as shown in Table 4. In both cases, the substrate-free control values were higher in the presence of O_2 in the gas phase and mitochondria

Table 4. *Incorporation of* [^{14}C]*leucine into liver and liver-tumour mitochondrial protein*

Mitochondria from liver or liver tumour were incubated with [^{14}C]leucine in the presence of cell sap from the same tissue for 50 min. at 37°. PEP refers to phosphoenolpyruvate and FDP to fructose diphosphate. The mitochondria were collected after incubation by centrifuging and the protein was precipitated with trichloroacetic acid. Radioactivity is expressed as in Table 1. Nicotinamide was added to inhibit the breakdown of diphosphopyridine nucleotide.

Additions	Gas phase	Radioactivity of protein: Liver	Radioactivity of protein: Liver tumour
None	N_2+CO_2 (95:5)	20	15
10 μmoles of PEP + 2 μmoles of ATP	N_2+CO_2 (95:5)	181	31
10 μmoles of FDP + 1 μmole of nicotinamide	N_2+CO_2 (95:5)	132	39
None	O_2+CO_2 (95:5)	44	33
10 μmoles of FDP + 1 μmole of nicotinamide	O_2+CO_2 (95:5)	229	66

Table 5. *Effect of fractions of the soluble cytoplasm on the incorporation of* [^{14}C]*leucine into mitochondria from normal liver and liver tumour*

Mitochondria were incubated with [^{14}C]leucine at 37° in N_2+CO_2 (95:5) for 50 min. The energy source was 10 μmoles of phosphoenolpyruvate (PEP) and 2 μmoles of ATP. Where the pH 5 fraction was used, PEP-kinase and 0·25 μmole of GTP were also included. In the absence of soluble fraction PEP-kinase was added. Radioactivity is expressed as in Table 1. Incorporation into cell sap alone is insignificant.

	Radioactivity of mitochondrial protein			
	Liver		Liver tumour	
Energy source	Absent	Present	Absent	Present
Soluble fraction present				
None	8	48	0	15
Liver whole cell sap	12	140	6	39
Tumour whole cell sap	11	153	9	43
Liver pH 5 fraction	6	88	—	—
Tumour pH 5 fraction	5	46	—	—

from both tissues exhibited the highest activity under aerobic conditions, with fructose diphosphate as substrate. The absolute values, however, were always lower in tumour preparations.

The results of the experiments on the effect of fractions of the cell sap on the incorporation of [^{14}C]leucine into hepatic mitochondria are summarized in Table 5. It can be seen that an energy-dependent incorporation of amino acid into the mitochondrial protein of both tissues occurred in the absence of cell sap, but the addition of cell sap to the incubation mixture stimulates this process. Moreover, the stimulating effect of tumour cell sap was at least as great as that of normal liver cell sap. The addition of the pH 5 fraction to liver mitochondria also results in an increased uptake of [^{14}C]leucine, but the pH 5 fraction of the tumour tissue is less active in this respect. The stimulating action of the pH 5 fraction was apparent only in the presence of GTP. It was observed, however, that the extent of incorporation which occurred in the absence of soluble fractions was not influenced by the presence of GTP.

Effect of ribonuclease on the amino acid incorporation into mitochondrial protein

Ribonuclease has been shown to inhibit the uptake of amino acids by microsomal preparations (Zamecnik & Keller, 1954) but not that into mitochondrial preparations (McLean *et al.* 1958). The effect of this enzyme on the incorporation of amino acids in mitochondria when incubated in the presence and absence of cell sap has now been studied. The results of a typical experiment are shown in Table 6. It can be seen that the incorporation in the absence of cell sap is stimulated by the lower concentration of ribonuclease, but is

Table 6. *Effect of ribonuclease on the incorporation of* [^{14}C]*leucine into liver mitochondria in the presence and absence of cell sap*

Mitochondria were incubated under N_2+CO_2 (95:5) at 37° for 50 min. with [^{14}C]leucine, 10 μmoles of phosphoenolpyruvate (PEP) and 2 μmoles of ATP, in the presence and absence of cell sap in the presence of different concentrations of ribonuclease. The mitochondria were then collected by centrifuging. The radioactivity of the mitochondrial protein is expressed as in Table 1. The control values, obtained in the absence of the PEP and ATP, did not exceed 8 counts/min.

Concn. of ribonuclease (mg./ml.)	Cell sap absent	Cell sap present
None	29	70
0·1	40	28
0·25	29	26

unaffected by the higher concentration. The incorporation of leucine into mitochondrial protein in the presence of cell sap, on the other hand, is reduced by both concentrations to the level obtained when cell sap is not included in the reaction mixture. Both in the presence and absence of cell sap the results obtained with 0·5 mg. of ribonuclease/ml. were the same as those with 0·25 mg./ml. Similar results were obtained with a different preparation of ribonuclease (Armour and Co. Ltd.).

DISCUSSION

Liver-tumour tissue is capable of a rapid rate of protein synthesis both *in vivo* and in the form of slices (Zamecnik *et al.* 1951; Campbell & Stone, 1957). The extent of the incorporation of amino acids into the microsomes of such slices is somewhat greater than that into the corresponding fraction of slices of normal liver (Campbell, Greengard & Jones, 1957). In spite of this, when microsomes were isolated from liver tumour and were incubated with radioactive amino acid and cell sap, the incorporation of radioactivity was much less than that in a similar system from normal liver. Efforts have been made to locate the reasons for this deficiency.

It was thought that the cell sap from the tumour might contain an inhibitor for the incorporation of amino acids into microsomes. Since the pH 5 fraction has been shown to replace the whole cell sap for incorporation of amino acids into normal liver microsomes, it was of interest to determine the effect of a similar fraction from tumour cell sap. However, the previously observed difference in the incorporation between the tumour and normal liver microsomes in the presence of whole cell sap was not altered by the substitution of pH 5 fraction.

On the basis that the ribonucleic acid in the pH 5 fraction, the so-called pH 5 RNA, plays a role in the transfer of amino acids to the microsomal protein, the incorporation of amino acids into pH 5 RNA from tumour cell sap was also studied. Less amino acid was incorporated into the pH 5 RNA derived from tumour than into that derived from liver, but the difference between the two ribonucleic acids was much less than the difference in activity between the pH 5 fractions for the incorporation of amino acids into the microsomes. It has previously been observed, in a comparison of regenerating and normal liver, that the ability of the pH 5 RNA to bind amino acid does not correlate with the ability of the pH 5 fraction to bring about incorporation into microsomes (Rendi, 1959). Thus it appears that the rate of transfer of amino acid from pH 5 RNA to the microsomes is more important than is the number of binding sites for amino acid on the pH 5 RNA.

Although the microsomes are the most active organelles of the liver cell for the incorporation of amino acid, and presumably for protein synthesis, an incorporation of amino acid dependent on an ATP-generating system has been obtained with mitochondrial preparations (Bates, Craddock & Simpson, 1958; Campbell & Greengard, 1959). It has been shown (Greengard, 1959) that even if the ribonucleic acid content of the mitochondria used in the present studies was due to contamination by microsomes, only a negligible fraction of the incorporated amino acid would have been due to the presence of microsomal protein. Similar considerations apply to the tumour mitochondria.

It will be seen from Table 4 that, in confirmation of the results of McLean *et al.* (1958) and Greengard (1959), normal liver mitochondria incorporate amino acids when supplied with a suitable source of energy. Liver-tumour mitochondria behave similarly, although the similarity is only qualitative, since the extent of incorporation of [^{14}C]leucine is lower than into the corresponding fraction of normal liver.

For the incorporation of amino acids into microsomal protein the cell sap is required (Zamecnik & Keller, 1954). However, for a similar incorporation into mitochondrial protein there appears to be no such requirement. Unless it is assumed that the constituents of the cell sap required for incorporation are retained within the membrane of the mitochondria, a different mechanism for the incorporation in the two types of organelle is indicated. The latter view is supported by the enhancement of the incorporation into mitochondria, in the absence of cell sap, by ribonuclease and the lack of effect of GTP, results which are contrary to experience with microsomes.

The increase in the incorporation of amino acids into mitochondria which is effected by the cell sap is reduced by the presence of ribonuclease (Table 6). The contrast between the effect of ribonuclease in the absence and presence of cell sap suggested that two mechanisms for the incorporation of amino acid might be operating. Although the second mechanism bears a certain resemblance to that which occurs with microsomes there do seem to be some differences. For mitochondria, pH 5 fraction (+GTP) probably does not include all the factors responsible for the stimulatory effect of cell sap (Greengard, 1959). The present studies with liver tumour are also of interest in this respect.

Liver-tumour cell sap or pH 5 fraction are much less effective than their normal counterparts in stimulating amino acid incorporation into microsomal protein; therefore if the same cell sap constituents were responsible for stimulating the process in both organelles, a similar quantitative difference would be expected in their effect on

mitochondrial incorporation. This, however, is not so (Table 5), since the cell sap derived from liver tumour was at least as effective as that derived from normal liver for the incorporation of amino acids into normal liver mitochondria. In contrast the tumour pH 5 fraction was considerably less effective than a similar fraction from normal liver in this respect. It thus appears that there are factors present in the cell sap which are not concerned with the incorporation of amino acids into microsomes, but which promote incorporation into mitochondrial protein.

SUMMARY

1. After the incubation of microsomes and pH 5 fraction of liver and liver tumour with [^{14}C]leucine, the radioactivity of the tumour-microsomal protein is lower than that of liver. When liver microsomes were incubated with tumour pH 5 fraction, instead of liver pH 5 fraction, a lower extent of incorporation resulted.

2. The adenosine triphosphate-dependent incorporation of leucine and lysine into the ribonucleic acid of pH 5 fraction of liver tumour was lower than into that of normal liver.

3. The adenosine triphosphate-dependent incorporation of [^{14}C]leucine into preparations of liver and tumour mitochondria was studied. Incorporation into the mitochondrial protein of both tissues took place in the absence and presence of cell sap, but was higher in its presence.

4. Under all experimental conditions the amino acid incorporation into tumour mitochondria was lower than into liver mitochondria. The stimulating action of tumour cell sap, however, on liver mitochondria was as great as that of liver cell sap. The tumour pH 5 fraction, on the other hand, was less effective than that of liver in stimulating mitochondrial incorporation.

5. Ribonuclease stimulates the incorporation into liver-mitochondrial protein in the absence of cell sap, but reduces the incorporation stimulated by cell sap.

We would like to thank Professor F. Dickens, F.R.S., for his encouragement and advice, and Miss B. Kernot and Miss P. Wilson for skilled technical assistance. The work was made possible by a grant to the Medical School by the British Empire Cancer Campaign.

REFERENCES

Bates, H. M., Craddock, V. M. & Simpson, M. V. (1958). *J. Amer. chem. Soc.* **80**, 1000.
Busch, H., Simbonis, S., Anderson, D. & Greene, H. S. N. (1956). *Yale J. Biol. Med.* **29**, 105.
Campbell, P. N. (1955). *Biochem. J.* **61**, 496.
Campbell, P. N. & Greengard, O. (1959). *Biochem. J.* **71**, 148.
Campbell, P. N., Greengard, O. & Jones, H. E. H. (1957). *Exp. Cell Res.* **12**, 689.
Campbell, P. N. & Stone, N. E. (1957). *Biochem. J.* **66**, 19.
Greengard, O. (1959). *Biochim. biophys. Acta*, **32**, 270.
Hoagland, M. B., Stephenson, M. L., Scott, J. F., Hecht, L. I. & Zamecnik, P. C. (1958). *J. biol. Chem.* **231**, 241.
Keller, E. B. & Zamecnik, P. C. (1956). *J. biol. Chem.* **221**, 45.
Littlefield, J. W. & Keller, E. B. (1957). *J. biol. Chem.* **224**, 13.
McLean, J. R., Cohn, G. L., Brandt, I. K. & Simpson, M. V. (1958). *J. biol. Chem.* **233**, 657.
Mejbaum, W. (1939). *Hoppe-Seyl. Z.* **258**, 117.
Ohlmeyer, P. (1951). *J. biol. Chem.* **190**, 21.
Rendi, R. (1959). *Biochim. biophys. Acta*, **31**, 266.
Schneider, W. C. & Hogeboom, G. H. (1950). *J. biol. Chem.* **183**, 123.
Zamecnik, P. C. & Keller, E. B. (1954). *J. biol. Chem.* **209**, 337.
Zamecnik, P. C., Loftfield, R. B., Stephenson, M. L. & Steele, J. M. (1951). *Cancer Res.* **11**, 592.

A Quantitative Method for the Separation of Thyroid Hormones and Related Compounds from Serum and Tissues with an Anion-Exchange Resin*

By VALERIE A. GALTON and ROSALIND PITT-RIVERS
National Institute for Medical Research, Mill Hill, London, N.W. 7

(*Received* 24 *October* 1958)

The use of paper chromatography and radioautography in the study of the metabolism of ^{131}I-labelled thyroid hormone is well known. Some concentration of the iodinated compounds present in biological material is usually necessary and the most widely used technique is that of extraction with butanol. However, the amount of material that can be extracted in this way is limited; this method is therefore not suitable when the amount of radioactivity is small, as chromatographic analysis of the extracted compounds is virtually impossible.

Blanquet, Dunn & Tobias (1955) have employed the anion-exchange resin Dowex-2 for the separation of organic and inorganic iodide present in hydrolysed thyroid gland or blood plasma; the

* This work forms part of a Ph.D. thesis submitted to the University of London.

resin was used in the hydroxyl form. Thyroxine, monoiodotyrosine and di-iodotyrosine were eluted in that order with 0·2N-hydrochloric acid; inorganic iodide remained on the resin throughout. Dowex-1 was equally effective and the two resins have been used in a study of thyroid function in the rat after administration of ^{131}I (Blanquet, Meyniel, Mounier, Stoll & Maraud, 1957). A 95% recovery of the iodinated compounds was effected by elution with 0·2N-hydrochloric acid containing 3·7% of ethanol (Meyniel, Blanquet, Mournier & Estibotte, 1958). However, relatively large volumes of hydrochloric acid were required to elute the iodinated amino acids.

Ingbar, Dowling & Freinkel (1957) used Dowex-1 equilibrated with acetate buffer at pH 4·5 for the purification of thyroxine-binding proteins. They found that thyroxine was dissociated from the plasma proteins by the resin; as the pH was reduced the thyroxine remained on the resin while all detectable amounts of protein were eluted, but the thyroxine could be quantitatively removed in concentrated solution by a further reduction in the pH of the eluent. The capacity of the resin for thyroxine was such that it could be concentrated from large volumes of serum. Further, as the solution of thyroxine obtained in this manner was free from protein or lipid material, it was suitable for paper chromatography.

The use of Dowex-1 was investigated for the separation, concentration and quantitative estimation of the thyroid hormones and related compounds from serum and tissues for chromatographic analysis. Preliminary experiments showed that thyroxine, which was absorbed by the resin at pH 4·5, could not be eluted with 0·1N-, 0·2N-, N- or 2N-hydrochloric acid, even after prolonged washing. It was, however, displaced with aqueous 40% (v/v) acetic acid at pH 1·6. Acetic acid solutions and acetate buffers were therefore used throughout this work.

METHODS AND MATERIALS

All experiments were carried out with a glass column of 1 cm. diam., containing a sintered-glass filter and the Dowex-1 resin: Dowex-1 R 1, X 2, 200–400 mesh, quaternary ammonium in chloride form.

Acetate buffers, pH 3·2–5·6, were made up from 0·2M-acetic acid and 0·2M-sodium acetate stock solutions; acetic acid was suitably diluted to give solutions of pH 1·4–3·0. The pH of all solutions was determined with a Cambridge pH meter.

Experiments were performed with serum to which had been added either thyroxine or tri-iodothyronine synthetically labelled with ^{131}I in the 3′ or 3′:5′ positions. The thyroxine and tri-iodothyronine samples were contaminated with iodide amounting to 12 and 15% respectively of the total ^{131}I in the samples.

A mixture of ^{131}I-labelled mono- and di-iodotyrosine was prepared from a mouse killed 4 hr. after injection of ^{131}I (10 μc). The thyroid gland was removed and was hydrolysed with trypsin in borate buffer at pH 8·6 for 24 hr. The hydrolysate was shown by chromatographic analysis to contain monoiodotyrosine, di-iodotyrosine, iodide and another iodinated fraction which remained at the origin of the chromatogram and was thought to be unhydrolysed or partially hydrolysed thyroglobulin.

The serum or thyroid hydrolysate containing one or more ^{131}I-labelled compounds was passed through the resin column (approx. 3 cm. high), which was equilibrated with buffer at pH 5·6. The resin was washed with the same buffer and then solutions of progressively decreasing pH were passed through the column, the solution being changed only when no further radioactivity could be eluted. Finally acetic acid, sometimes followed by 3N-NaBr to elute the iodide, was passed through the column. The eluate was collected from the column in 3 ml. serial fractions. The total radioactivity in the original samples and in the fractions eluted from the column was estimated in a Well-type scintillation counter. When necessary, a sample was counted in a total volume of 3 ml.

In order to determine whether a compound was eluted quantitatively from the column, one-dimensional chromatographic analysis of the iodinated compounds present in the original sample and in the fractions of eluate from the column was carried out. The solvent systems butan-1-ol–dioxan–aq. 2N-NH_3 soln. (4:1:5) and butan-1-ol–2N-acetic acid (1:1) were used throughout. Synthetic mono- and di-iodotyrosine, thyroxine and tri-iodothyronine were employed as markers and were stained by the method of Gross & Leblond (1951). Carrier iodide was stained with 0·1% of palladium chloride. The chromatograms were counted with an automatic recording ratemeter and the area under each peak was measured with a planimeter; the area is directly proportional to the radiation emitted from the corresponding part of the chromatogram. These measurements enable the distribution of the total radioactivity among the different compounds present on a chromatogram to be calculated. As the total radioactivity in the original sample and in the separate fractions from the column was also determined it was possible to trace quantitatively the fate of each compound.

When the radioactivity of a protein-containing fraction was very low, the solution was first exhaustively extracted with butan-1-ol and the concentrated extracts were analysed. Protein-free solutions were evaporated to dryness under reduced pressure and the residues were dissolved in methanol–aq. NH_3 soln. (sp.gr. 0·880) (3:1). Chromatographic analysis of the fraction eluted with NaBr was seldom possible since the NaBr was precipitated on concentration.

In a further experiment the kidneys of a normal mouse were removed, 0·9% NaCl soln. was added (6 ml.) and the tissues were homogenized with sand in a pestle and mortar and centrifuged. [^{131}I]Thyroxine was added to the extract in 0·9% NaCl soln. and the resulting solution was subjected to analysis on a column of Dowex-1.

RESULTS

Thyroxine. The results are shown in Table 1. As the serum passed through the column, 100% of the radioactivity present in the serum was absorbed.

Radioactivity amounting to 84% of that in the original sample was discharged from the column with 40–50% acetic acid (pH 1·4–1·6) and was identified by chromatographic analysis as thyroxine. The remaining activity was eluted with NaBr. The concentration of radioactivity in this fraction was too low to permit chromatographic analysis, but it was assumed to consist of iodide since 16% of the radioactivity in the original sample was in the form of inorganic iodide which had not been detected in the fraction eluted from the column at pH 1·4.

Tri-iodothyronine. The results are shown in Table 2. All the serum radioactivity was absorbed by the resin. (In all experiments, at least 96% of the radioactivity was absorbed.) Radioactivity (84·5%) was discharged with 30–50% acetic acid (pH 1·8–1·4) and identified by chromatographic analysis as tri-iodothyronine. The remaining 15% was removed with 3N-NaBr and was sufficiently active to permit chromatographic analysis; it consisted entirely of iodide and presumably represented the iodide present to the extent of 15% of total radioactivity in the original serum.

Monoiodotyrosine, di-iodotyrosine and thyroglobulin. The thyroid hydrolysate containing these compounds and iodide was analysed on the column. The results are shown in Table 3. Chromatographic analysis of the three principal fractions eluted from the column indicated that separation was complete. The distribution of the total radioactivity among the three main components of the hydrolysate was calculated from chromatographic analysis and column analysis (Table 3) and the results obtained were compared (Table 4). The monoiodotyrosine/di-iodotyrosine ratio determined by chromatography was 2·7; after separation on the resin it was 2·8. It was concluded that mono- and di-iodotyrosine were absorbed by Dowex-1 and quantitatively eluted with 1·15 and 10% acetic acid respectively.

Sodium chloride solution extract of kidney homogenate containing thyroxine. [^{131}I]Thyroxine (1 μc of ^{131}I) was added to an extract of kidney homogenate in 0·9% NaCl soln. The solution was poured on the top of a Dowex-1 column; it passed down the column at first but gradually the rate of flow diminished and finally stopped. However, under pressure (equal to 20 cm. water) the solution slowly resumed its passage through the resin. Under these conditions 96% of the radioactivity was absorbed. Although most of the tissue proteins passed unhindered through the column, prolonged washing

Table 1. *Separation of thyroxine from serum with Dowex-1*

Fraction	pH or solvent	Counts/min.	^{131}I-labelled compounds detected by chromatography
Serum + thyroxine	7·4	15 468	Thyroxine, iodide
1*	5·6	654	Thyroxine, iodide
2–9	5·6–1·8	—	—
10	1·67	641	
11	1·4	5 744	Thyroxine
12	1·4	5 959	
13	1·4	589	
14–20	Undiluted acetic acid	—	—
21–30	3N-NaBr	1 900	—
Total eluted material		15 487	

* Fraction containing most of original protein.

Table 2. *Separation of tri-iodothyronine from serum with Dowex-1*

Fraction	pH or solvent	Counts/min.	^{131}I-labelled compounds detected by chromatography
Serum + tri-iodothyronine	7·4	751 960	Tri-iodothyronine, iodide
1*	5·6	1 803	Tri-iodothyronine, iodide?
2*	5·6	1 716	
3–11	5·6–2·0	0–50	—
12	1·8	681	
13	1·67	440 310	
14	1·4	164 640	Tri-iodothyronine
15	1·4	29 500	
16	1·4	500	
17–25	Undiluted acetic acid	—	—
26–40	3N-NaBr	112 860	Iodide
Total eluted material		752 060	

* These fractions contained most of the original protein.

Table 3. *Separation of iodinated amino acids in a thyroid hydrolysate with Dowex-1*

Fraction	pH or solvent	Counts/min.	^{131}I-labelled compounds detected by chromatography
Thyroid hydrolysate	8·6	30 420	Thyroglobulin, monoiodotyrosine, di-iodotyrosine, thyroxine, iodide
1–5	5·6	—	
6–10	3·6	800	Thyroglobulin
11–16	3·4–3·2	—	—
17–24	3·0	20 447	Monoiodotyrosine
25–35	2·8–2·4	—	—
36–40	2·2	7 371	Di-iodotyrosine
41–100	1·6–1·4	300	Thyroxine?
101–120	3N-NaBr	1 102	Iodide
Total eluted material		30 020	

Table 4. *Percentage distribution of total ^{131}I among the different iodinated compounds in the thyroid hydrolysate as determined by two methods*

Compound	Paper-chromatographic analysis	Column analysis
Thyroglobulin	5	3
Monoiodotyrosine	65	67
Di-iodotyrosine	24	24
Other compounds	6	5

with buffer at pH 3·6 was necessary before the eluates were free from any detectable amount of protein. Thyroxine remained on the column throughout and was quantitatively eluted with 50% acetic acid. It was observed that as the flow rate of the protein solution through the column was increased by raising the pressure, the efficiency of the resin in extracting the radioactive thyroxine was reduced.

DISCUSSION

It is evident from these experiments that thyroxine, tri-iodothyronine, mono- and di-iodotyrosine and thyroglobulin can be separated and concentrated from serum and tissues by absorption on Dowex-1. This technique has several advantages over that of butanol extraction. The iodinated compounds are separated from the proteins and lipid materials of the tissue; they are eluted with a volatile solvent which can easily be removed. The residue, on treatment with methanol–ammonia, gives a non-viscous extract which can be cleanly applied to filter paper for chromatographic analysis.

The capacity of the resin for thyroxine and its analogues is sufficient to permit their removal from much greater amounts of material than can be employed for butanol extraction. The technique therefore makes possible the study of thyroid-hormone metabolism in the tissues of animals given quite small doses of ^{131}I.

Inorganic and organic iodide present in a tissue can be accurately estimated by using Dowex-1. The protein-bound ^{131}I present in serum 24 hr. after a mouse had received 10 μc of ^{131}I was determined (*a*) by chromatographic analysis, (*b*) by column analysis and (*c*) by trichloroacetic acid precipitation. Chromatographic and column analyses gave comparable results, whereas a considerably higher value for protein-bound ^{131}I was obtained by trichloroacetic acid precipitation unless a lengthy procedure of washing, re-dissolving in alkali and re-precipitation was adopted.

Dowex-1 can therefore be used to advantage in the study of quantitative aspects of thyroid-hormone metabolism in various tissues and body fluids.

SUMMARY

1. The thyroid hormones and related compounds can be separated from biological material by absorption on the ion-exchange resin Dowex-1 equilibrated with acetic acid–sodium acetate buffer at pH 5·6.

2. The iodinated compounds are quantitatively eluted when the pH is reduced with acetic acid solutions of increasing concentration.

3. Thyroglobulin, monoiodotyrosine and di-iodotyrosine are eluted at pH 3·6, 3·0 and 2·2 respectively and thyroxine and tri-iodothyronine are eluted at pH 1·4. Iodide remains on the resin throughout.

4. Dowex-1 can therefore be used to concentrate the thyroid hormones in biological material for chromatographic analysis.

REFERENCES

Blanquet, P., Dunn, R. W. & Tobias, C. A. (1955). *Arch. Biochem. Biophys.* **58**, 502.

Blanquet, P., Meyniel, G., Mounier, J., Stoll, R. & Maraud, R. (1957). *Ann. Endocr., Paris*, **18**, 864.

Gross, J. & Leblond, C. P. (1951). *Endocrinology*, **48**, 714.

Ingbar, S. H., Dowling, J. T. & Freinkel, N. (1957). *Endocrinology*, **61**, 321.

Meyniel, G., Blanquet, P., Mounier, J. & Estibotte, M. (1958). *Bull. Soc. Chim. biol., Paris*, **40**, 369.

Thyroid-Hormone Metabolism in the Kidney*

By VALERIE A. GALTON and ROSALIND PITT-RIVERS
National Institute for Medical Research, Mill Hill, London, N.W. 7

(*Received* 24 *October* 1958)

Present knowledge of the metabolism of the thyroid hormones in the kidney is confined mainly to information obtained from experiments with ^{131}I-labelled thyroxine and tri-iodothyronine. Gross & Leblond (1951*a*) found that after the administration of [^{131}I]thyroxine to rats or mice, the kidney concentrated radioactivity from the blood. Subsequent chromatographic analysis of butanol extracts of kidney revealed the presence of at least four ^{131}I-labelled compounds: thyroxine, tri-iodothyronine, iodide and an unknown compound which was later shown to give rise to thyroxine on alkaline hydrolysis (Leblond & Cambron, 1952). The compound was also detected in bile and was thought to be a conjugate of thyroxine with glucuronic acid. Gross, Ford, Symchowicz & Horton (1957) found that [^{131}I]tri-iodothyronine when administered to rabbits gave rise to a metabolite in the kidney, plasma and liver which yielded tri-iodothyronine on hydrolysis with 0·2N-hydrochloric acid. The liver also contained a second conjugated compound which they identified as tri-iodothyronine glucuronide. Roche, Michel, Jouan & Wolf (1956) have shown that the kidney can deiodinate and deaminate [^{131}I]tri-iodothyronine, forming 3:3′-di-iodothyronine and tri-iodothyroacetic acid [4-(4-hydroxy-3-iodophenoxy)-3:5-di-iodophenylacetic acid].

Results obtained in experiments with synthetic thyroid hormones do not necessarily reflect the normal metabolism of endogenous thyroid hormone. A satisfactory qualitative and quantitative study of thyroid-hormone metabolism in the kidney has not yet been carried out. For this it is essential to label the endogenous thyroid hormones with ^{131}I; further, an analysis of the iodinated compounds present in the kidney must be carried out only after the administration of tracer doses of ^{131}I, in order to avoid radiation damage to the thyroid gland sufficient to cause the release of thyroglobulin into the circulation. The kidney is primarily an excretory organ and would be expected to partake in the removal of any abnormal iodinated compounds present in the circulation. Fletcher (1957) detected a number of compounds in the urine but not in the serum of patients treated with large doses of ^{131}I for thyroid carcinoma, and he suggested that they may be breakdown products of the thyroglobulin which was present in the serum of these patients. The maximum total ^{131}I in the kidneys after tracer doses of ^{131}I is such that it is impossible to detect and identify satisfactorily the iodinated compounds present in a butanol extract of the tissue. However, by absorption on Dowex-1 it is possible to separate and concentrate the iodinated compounds from a sufficient amount of tissue to permit their detection by chromatographic analysis (Galton & Pitt-Rivers, 1959).

The present work describes how this resin was used in a qualitative and quantitative study of thyroid-hormone metabolism in the kidneys of mice given very small doses of ^{131}I.

METHODS AND MATERIALS

Preparation of tissue for analysis. Six male albino mice (20 g.) fed on diet 41 of Bruce & Parkes (1946) were used in each experiment. Each mouse received ^{131}I (10–20 μc) intraperitoneally and was killed 60–72 hr. later by exsanguination under ether anaesthesia. Urine was taken from the bladders with a syringe. The kidneys were removed, rapidly skinned and weighed, rinsed in ice-cold 0·9% NaCl soln. and homogenized in a pestle and mortar with thiouracil (2 mg.), 0·9% NaCl soln. (6 ml.) and a little acid-washed sand. Homogenization was carried out slowly with the mortar standing in an ice–salt freezing mixture. The homogenate was transferred to centrifuge tubes, a further 14 ml. of 0·9% NaCl soln. was added and the mixture was shaken and centrifuged, the temperature being kept below 5°. The volume of the extract in 0·9% NaCl solution was measured and a portion was estimated for radioactivity in a well-type scintillation counter.

Column analysis. The experiments were carried out at 4°. Dowex-1 was equilibrated with sodium acetate buffer at pH 5·6. Sodium metabisulphite (0·02 mg./ml.) was added to all the eluents to prevent any oxidation of iodide on the column. The kidney extract was passed through the column (3 cm., 1 cm. diameter) of resin four times. The column was then washed with buffer at pH 5·6 until the eluate contained no detectable amount of protein, followed by buffer at pH 3·6, acetic acid solutions at pH 3·0, 2·2 and 1·4, undiluted acetic acid and finally 3N-NaBr. At least 10 ml. of each solution was passed through the column. The eluates were collected into 10 ml. graduated centrifuge tubes and samples were estimated for radioactivity. The resin itself was counted in the scintillation counter in order to be sure that all the radioactivity had been discharged.

* This work forms part of a Ph.D. thesis submitted to the University of London.

Column analysis of the urine and serum was carried out in a similar manner.

Chromatographic analysis. A sample of urine was chromatographically analysed. The eluates containing radioactivity were pooled, evaporated to dryness under reduced pressure and the remaining acetic acid was removed in a stream of air. The residues were dissolved in methanol–aq. NH_3 soln. (sp.gr. 0·88) (3:1) and analysed by paper chromatography as described by Galton & Pitt-Rivers (1959). The solvent systems used were butan-1-ol–dioxan–aq. 2N-NH_3 soln. (4:1:5); butan-1-ol–2N-acetic acid (1:1); *tert.*-amyl alcohol–aq. 2N-NH_3 soln. (1:1). Carriers were used for identification and stained by the method of Gross & Leblond (1951*b*). The chromatograms were scanned with a strip counter or radioautographed on X-ray film.

Identification of the unknown metabolite

Preparation of compound. In order to increase the specific activity of the metabolite the mice used in these experiments were given 200 μc of ^{131}I. The kidneys from six mice were analysed on the column as previously described. The fraction eluted with 50% acetic acid (pH 1·4) was evaporated to dryness as previously described and the residue was dissolved in 0·1 ml. of methanol–aq. NH_3 soln. This solution was chromatographed on paper in butanol–dioxan–aq. NH_3 soln. The area containing the metabolite was located with an end-window Geiger–Müller tube, removed and cut into pieces.

Hydrolysis of metabolite. The paper containing the metabolite was placed in a counting tube and 0·3 ml. of the acid, alkali or buffer solution to be used was added. The paper was removed from the liquid after 1 min., squeezed well with forceps and lifted out of the tube. The ^{131}I in the tube was estimated in the scintillation counter before and after the paper had been removed to check that satisfactory elution had been achieved; it was invariably found that 90–95% of the total ^{131}I on the paper was eluted by this technique. Attempts to hydrolyse the compound were made with 2N-HCl at 100° for 18 hr., 5N-HCl at 100° for 1 hr. (SO_2 was bubbled through the 5N-HCl during hydrolysis to prevent oxidative destruction) and N-NaOH at 100° for 1 hr.

A crude preparation of β-glucuronidase was made by suspending scrapings of the mucosa of the large intestine of a rat in a few drops of 0·9% NaCl soln. Incubation of the metabolite was carried out in 0·1M-sodium phosphate buffer at pH 6·0, with 1 drop of this suspension, at 37° for 1, 4 or 18 hr.

Three arylsulphatases, free from β-glucuronidase, were incubated with the metabolite at 37° for 4 or 24 hr. These were a bacterial sulphatase from *Alcaligenes metalcaligenes*, in 0·1M-2-amino-2-hydroxymethylpropane-1:3-diol–HCl buffer at pH 9·0, a limpet sulphatase in 0·2M sodium acetate buffer at pH 5·5 and a rat-liver sulphatase in 0·1M-sodium phosphate buffer at pH 8·0. The ^{131}I-labelled compounds present in the incubation media or in the acid and alkali solutions were determined by chromatographic analysis.

Experiments with ^{35}S-labelled sulphate

The metabolite was prepared from the kidneys of six mice injected with ^{35}S-labelled sulphate in addition to 200 μc of ^{131}I; carrier-free $Na^{35}SO_4$ (250 μc in 0·5 ml. of 0·9% NaCl soln.) was injected twice subcutaneously into each mouse, 24 and 16 hr. before they were killed. The metabolite, purified by chromatography, was re-chromatographed in butanol–dioxan–aq. NH_3 soln. and the chromatogram was counted twice with a strip counter; the first time the end window was uncovered and the second time it was shielded with aluminium foil (11·4 mg./cm.^{2}). This shield will absorb all the ^{35}S radiation but allows about 60% of the ^{131}I radiation to pass through (J. R. Tata, personal communication). For comparative purposes this technique was carried out on a chromatogram containing only a single radioactive spot of ^{131}I. The proportion of radioactivity absorbed by the aluminium was determined by measuring the area under the peaks on the two records obtained from each chromatogram with a planimeter. The metabolite in this experiment was hydrolysed with the intestinal β-glucuronidase and the products of hydrolysis were analysed by chromatography to determine whether ^{35}S-labelled sulphate was released.

RESULTS

The 0·9% NaCl soln. extract (19·4 ml.) from six kidneys (2·77 g.) contained at least 98% of the total ^{131}I. It was evident from the column analysis of the kidney extract (Table 1) that thyroglobulin, mono- or di-iodotyrosine, which would have been discharged from the resin by acetate buffer at pH 3·6 and acetic acid solutions at pH 3·0 and 2·2, were not present. Two iodinated compounds, one of which was identified as thyroxine, were detected by chromatographic analysis of the fraction eluted with 50% acetic acid (pH 1·4) (Figs. 1 and 2). Most of the ^{131}I in this fraction (80–95%) was in the form of a metabolite which could not be identified with any synthetic thyroxine analogue available. On some of the radioautographs a trace of radioactivity corresponding to tri-iodothyronine was detected. The amount of organically bound ^{131}I present varied between 26 and 70% of the total ^{131}I in the kidney solution. The highest value was always obtained in the mice killed between 60 and 72 hr. after the injection of ^{131}I.

The efficiency of the resin in absorbing the radioactivity from the saline solution was considerably less than that observed when only thyroxine and iodide were present (Galton & Pitt-Rivers, 1959). In order to determine the nature of the unabsorbed ^{131}I-labelled compounds, six mice were each given 200 μc of ^{131}I. They were killed 72 hr. later and the kidneys were analysed on the column. The protein solution was passed through the column four times and the radioactivity which remained in the solution was investigated by butanol extraction followed by chromatographic analysis. It was found that practically all the ^{131}I was in the form of the unidentified metabolite described above (Fig. 2); only a trace amount of radioactivity corresponding to thyroxine was detected and this was of doubtful significance. In this experiment

Table 1. *Column analysis of extract of kidney in sodium chloride solution*

Fraction	pH or solvent	Volume	Total counts/min.	Percentage of total ^{131}I
Kidney extract	7·4	19·4	28 984	100
1*	5·6	10·2	1 999	15·5
2	5·6	8·3	2 424	
3	5·6	10·3	—	—
4	3·6	10·0	—	—
5	3·0	10·0	—	—
6	2·2	10·0	—	—
7	2·2	6·0	—	—
8	1·4	10·3	8 240	54
9	1·4	12·0	5 796	
10	1·4	7·4	1 325	
11	1·4	7·4	296	
12	3N-NaBr	30·0	8 820	30·5
	Total eluted material		28 900	

* Fraction containing most of the original protein, etc.

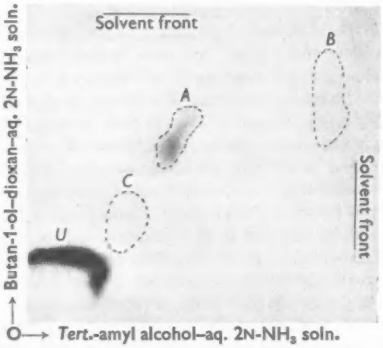


Fig. 1. Radioautograph of two-dimensional chromatogram prepared from kidney fraction eluted at pH 1·4. Solvent systems: butan-1-ol–dioxan–aq. 2N-NH_3 soln. (4:1:5) and *tert.*-amyl alcohol–aq. 2N-NH_3 soln. (1:1). *A*, Thyroxine; *B*, tri-iodothyronine; *C*, iodide; *U*, unknown metabolite.

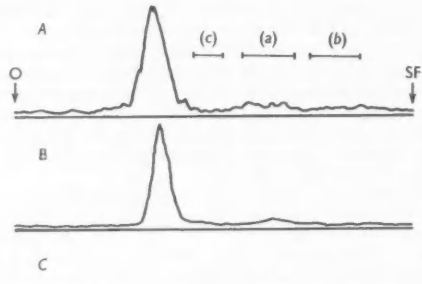


Fig. 2. Scans of radioactive chromatograms prepared from *A*, butan-1-ol extract of kidney solution from column; *B*, kidney fraction eluted from column at pH 1·4; *C*, urine; *D*, serum fraction eluted at pH 1·4. Solvent system: butan-1-ol–dioxan–aq. 2N-NH_3 soln. (*a*) Thyroxine; (*b*) tri-iodothyronine; (*c*) iodide. O, Origin; SF, solvent front. Maximum scale represents about 0·005 μc of ^{131}I.

the concentration of radioactivity in the fraction eluted with NaBr was high enough to permit chromatographic analysis; the ^{131}I was entirely in the form of inorganic iodide.

Column analysis of serum was also carried out (Table 2). No radioactivity was detected in the fraction eluted with buffer at pH 3·6. This indicates that thyroglobulin was not present in the serum. Chromatographic analysis of the radioactive material eluted with 50 % acetic acid revealed that only thyroxine was present; there was no evidence that the unidentified metabolite present in kidney occurred in serum (Fig. 2).

All detectable radioactive material in the urine was absorbed by the resin and could be eluted only with NaBr. Chromatographic analysis of a sample of urine confirmed that all the radioactivity was in the form of inorganic iodide; clearly the metabolite detected in the kidney was not secreted into the urine.

The ratio organic ^{131}I/inorganic ^{131}I was 2·3 in the kidney and 1·8 in the serum. These values were calculated from column analyses of serum and tissues from the same group of mice (Tables 1 and 2). In another experiment the total ^{131}I concentration in the kidneys and serum was about equal. The radioactivity in the urine was about 100 times

Table 2. *Column analysis of serum*

Fraction	pH or solvent	Volume	Total counts/min.	Percentage of total ^{131}I
Serum	7·4	2·47	23 275	100
1*	5·6	10·0	1 190	5
2	3·6	10·0	—	—
3	3·0	10·0	—	—
4	2·2	10·0	—	—
5	1·4	9·6	8 275	
6	1·4	7·7	3 873	59
7	1·4	7·3	1 533	
8	3N-NaBr	30·0	8 310	36
	Total eluted material		23 181	

* Fraction containing most of the original protein, etc.

as high as in either serum or kidney and consisted entirely of iodide; since small amounts of urine are always present in kidney extracts this would give a falsely high value for kidney inorganic ^{131}I. However, in spite of this higher proportion of organic iodine, it was evident that the concentration of free thyroxine in the kidney was small compared with that in the serum.

Identification of unknown metabolite

2N-Hydrochloric acid had little or no hydrolytic effect on the metabolite even at 100° for 18 hr. However, with 5N-HCl the metabolite was hydrolysed completely in 1 hr. When the hydrolysate was analysed by chromatography, only thyroxine was detected. The metabolite was also hydrolysed with N-NaOH but some deiodination of the liberated thyroxine also occurred. Hydrolysis of the metabolite was achieved within 1 hr. by the crude intestinal-enzyme preparation, but the three sulphatases had no effect on the compound; no thyroxine was detected in the media even after incubation for 24 hr.

From the control chromatogram it was estimated that the aluminium shield covering the end window of the strip counter absorbed 40 % of the ^{131}I radiation. When a chromatogram containing the metabolite prepared from mice which had received ^{131}I and $^{35}SO_4^{2-}$ was scanned with and without this filter, only 40 % of the total radiation was absorbed, indicating that the metabolite contained no significant amount of sulphur. This conclusion was confirmed in the hydrolysis experiment. When the hydrolysate was chromatographically analysed, only one radioactive spot which corresponded to carrier thyroxine was detected; there was no evidence of any free radioactive sulphate.

DISCUSSION

The experiments described above indicate that at least 98 % of the total iodine present in the kidney is distributed among three substances: iodide, thyroxine and a third compound not previously identified in this organ. Tri-iodothyronine was occasionally found, but only in very small amounts. Although 60–70 % of the total iodine in the kidney is organically bound only 5–10 % of this is in the form of free thyroxine; at least half of the kidney iodine is in the form of the unknown metabolite. These estimations are subject to error owing to the presence of urine in the kidney tubules; it is possible that the ratio of organic to inorganic iodine in the kidney cells is higher than was estimated here.

It was concluded that these results reflect the normal metabolism of iodine in the kidney because, first, the dose of ^{131}I used was small and radiation damage to the thyroid was not likely. The results obtained from the analysis of serum supported this view; no thyroglobulin was detected. Secondly, it was observed that the ratio of organic to inorganic ^{131}I in the kidney did not reach a maximum until between 50 and 65 hr. after administration of ^{131}I; after this interval the ratio remained approximately constant. This was taken as an indication that equilibration between the ^{131}I and ^{127}I in the kidney had been reached. Thirdly, the technique permits a quantitative analysis of at least 98 % of the total ^{131}I present in the kidney; in the kidney tissue the amount of radioactive material which was insoluble in sodium chloride solution was small and the resin showed no detectable radioactivity after the iodide had been eluted with sodium bromide solution.

The possibility that the metabolite was an artifact produced during the analytical procedure was eliminated as it was not found in serum or urine which were subjected to the same procedure. Further, the metabolite was detected in the kidneys of mice treated with larger doses of ^{131}I which were not analysed on the column but were extracted with butanol.

On hydrolysis the metabolite yielded thyroxine. It was rapidly hydrolysed when incubated with scrapings of the mucosa of rat large intestine which contains both β-glucuronidase and sulphatases

among other enzymes. It was not possible to obtain β-glucuronidase in a pure state free from sulphatase activity, but the sulphatases used were freshly prepared and stated to be free from β-glucuronidase (K. S. Dodgson, personal communication). None of the three sulphatases was capable of hydrolysing the metabolite. These enzymes are known to hydrolyse tyrosine-*O*-sulphate and some of its derivatives but since there was no direct evidence that thyroxine-*O*-sulphate would be hydrolysed by the sulphatases, these experiments do not provide unequivocal proof that the metabolite is not the ethereal sulphate of thyroxine. However, the experiments carried out on the metabolite prepared from mice treated with both $^{35}SO_4^{2-}$ and ^{131}I substantiated the view that the metabolite did not contain sulphur.

It is now generally accepted that the thyroid hormones undergo conjugation with glucuronic acid in the liver and are excreted as such a conjugate in the bile. It is not unreasonable to suppose that the kidney contains a similar mechanism for conjugating thyroid hormones. It was therefore concluded from the results obtained in the present work that the kidney metabolite is a conjugate of thyroxine with glucuronic acid. This compound is probably identical with that detected by Gross & Leblond (1951*b*) in the kidneys of mice treated with [^{131}I]thyroxine; their metabolite was stable to N-hydrochloric acid but yielded thyroxine on alkaline hydrolysis. The absence of the metabolite from the serum and urine indicates that both its formation and metabolic breakdown must take place in the kidney tissue itself.

The concentration of organic iodine though not that of unconjugated thyroxine is approximately the same in the kidney and serum. This contrasts with the finding of Tata (1958) that muscle and brain contain comparatively low concentrations of organic iodine. Further, there is no evidence that conjugation of the thyroid hormones takes place in these tissues. Tata (1958) has suggested that the transport of thyroxine from the serum into the cells is dependent on the different thyroxine-binding properties of serum, extracellular and intracellular proteins. This results in a high serum/tissue thyroxine concentration ratio. In the kidney, however, the glucuronide-forming mechanism may compete with the proteins for the intracellular thyroxine with the result that some thyroxine is dissociated from the proteins and conjugated, thus permitting more thyroxine to pass into the cell than would otherwise be possible. There is no reason to suppose that the kidney is dependent for normal function on a concentration of thyroxine many times that in muscle and brain. It is more likely that this conjugating system is part of a mechanism whereby the kidney assists in the regulation of blood thyroxine concentration; the glucuronide undergoes metabolic breakdown without having exerted any biological effect, and the physiological action of the thyroid hormone is brought about by the trace amounts of free thyroxine in the kidney. The way in which thyroxine glucuronide is metabolized is unknown; the assumption that it is physiologically inert, which forms the basis of these suggestions, has yet to be justified.

SUMMARY

1. The anion-exchange resin Dowex-1 was used to separate the iodinated compounds from the kidneys of mice treated with small doses of ^{131}I.

2. 60–70% of the ^{131}I in the kidney after 70 hr. was organically bound.

3. Chromatographic analyses of the organic iodine compounds after elution indicated that only small amounts of thyroxine were present.

4. Most of the organic ^{131}I occurred in a metabolite of thyroxine which was shown to be a conjugate. This metabolite was not present in the serum or urine.

The authors would like to express their thanks to Dr K. S. Dodgson for the gift of the arylsulphatases used in this work.

REFERENCES

Bruce, H. M. & Parkes, A. S. (1946). *J. Hyg., Camb.*, **44**, 501.

Fletcher, K. (1957). *Biochem. J.* **67**, 140.

Galton, V. A. & Pitt-Rivers, R. (1959). *Biochem. J.* **72**, 310.

Gross, J., Ford, D. F., Symchowicz, S. & Horton, J. H. (1957). *Ciba Foundation colloq. Endocrinol.* **10**, 182.

Gross, J. & Leblond, C. P. (1951*a*). *Proc. Soc. exp. Biol., N.Y.*, **761**, 686.

Gross, J. & Leblond, C. P. (1951*b*). *Endocrinology*, **48**, 714.

Leblond, C. P. & Cambron, J. (1952). *Anat. Rec.* **112** (suppl.), 448.

Roche, J., Michel, R., Jouan, P. & Wolf, W. (1956). *Endocrinology*, **59**, 425.

Tata, J. R. (1958). *Biochim. biophys. Acta*, **28**, 91.

The Identification of the Acetic Acid Analogues of Thyroxine and Tri-iodothyronine in Mammalian Tissues*

By VALERIE A. GALTON and ROSALIND PITT-RIVERS
National Institute for Medical Research, Mill Hill, London, N.W. 7

(*Received* 24 *October* 1958)

The acetic acid analogues of thyroxine and tri-iodothyronine, namely tetraiodothyroacetic acid [4-(4′-hydroxy-3′:5′-di-iodophenoxy)-3:5-di-iodophenylacetic acid] and tri-iodothyroacetic acid [4-(4′-hydroxy-3′-iodophenoxy)-3:5-di-iodophenylacetic acid] have been synthesized (Harington & Pitt-Rivers, 1952; Pitt-Rivers, 1953) and shown to possess considerable biological activity when assayed by the goitre-prevention test in rats (Pitt-Rivers, 1953). Pitt-Rivers suggested that tetra- and tri-iodothyroacetic acids might arise during the peripheral metabolism of the thyroid hormones by oxidative deamination and decarboxylation of the alanine side chain of the parent amino acid. In support of this hypothesis Roche, Michel & Tata (1954) found that rats injected with large doses of tetra- or tri-iodothyroacetic acid excrete the corresponding pyruvic acid analogue in their urine and bile. Traces of tri-iodothyroacetic acid have been detected in the kidney and muscle of rats given small doses tri-iodothyronine (Roche, Michel, Jouan & Wolf, 1956), and tetraiodothyroacetic acid was found in the serum of dogs injected with [^{131}I]-thyroxine (Flock, Bollman, Grindlay & McKenzie, 1957). Tomita, Lardy, Larson & Albright (1957) detected tetra- or tri-iodothyroacetic acid in rat-kidney homogenates or cell-free extracts after incubation with thyroxine or tri-iodothyronine. Tata has obtained evidence that brain and muscle slices and homogenates can convert thyroxine and tri-iodothyronine into their respective acetic acid analogues (Tata, 1957; Tata, Rall & Rawson, 1957). It therefore appears that certain tissues can convert thyroxine and tri-iodothyronine into the corresponding acetic acid analogues, but it does not necessarily follow that this is a normal metabolic process. The experiments described below were carried out to determine whether the thyroacetic acids are formed from endogenous thyroid hormones after the administration of radioactive iodide.

METHOD AND MATERIALS

Three male albino mice (20 g.) were used for each experiment; the animals were fed for 3 weeks before the experiment on an iodine-poor diet consisting of wholemeal flour (88% extracted) 1000 parts, gluten 660 parts, I.C.F. 200 parts and yeast 140 parts (I.C.F., $CaHPO_4$ 40 parts, $FeSO_4,7H_2O$ 2 parts and flour 200 parts). Deionized water was administered *ad lib.* Animals were given a single intraperitoneal injection of ^{131}I (1 mc) and groups were killed by exsanguination under ether anaesthesia 12–48 hr. later.

Preparation of tissue extracts. The liver and kidneys were removed, freed from surrounding tissues, rinsed rapidly in ice-cold 0·9% NaCl soln. and frozen in a beaker in dry ice. After partial thawing, tissues were cut into small pieces, homogenized in metal-free glass and Teflon homogenizers immersed in a freezing mixture and transferred to ice-cold centrifuge tubes with a little 0·9% NaCl soln. The pH of the homogenates was reduced to 2 with 5N-HCl and they were extracted five times with equal volumes of butan-1-ol. The pooled extracts were evaporated to dryness under reduced pressure below 40°; the residues were transferred to centrifuge tubes with four 0·5 ml. portions of methanol–aq. NH_3 soln. (sp.gr. 0·88) (3:1). After centrifuging, the supernatant liquids, which contained nearly all of the radioactivity, were concentrated in a stream of air and recentrifuged. The final supernatant solution was used for chromatographic analysis.

Chromatographic analysis. Ascending chromatography on Whatman no. 1 paper was used throughout. Solvent systems were butan-1-ol–dioxan–aq. 2N-NH_3 soln. (4:1:5) and *tert.*-amyl alcohol–aq. 2N-NH_3 soln. (1:1). The dioxan was purified by Vogel's (1946) method. Thiouracil (0·01 mg./ml.) was added to the solvent systems to prevent any oxidation of iodide during chromatography. The butanol extracts contained, in addition to the iodinated compounds, fat and lipid materials from the homogenized tissues, which made chromatographic analysis difficult. The following technique was therefore adopted. Preliminary one-dimensional chromatograms of the tissue extracts were developed in both butanol–dioxan–aq. NH_3 soln. and *tert.*-amyl alcohol–aq. NH_3 soln. Separate spots, 0·5 cm. in diameter, were applied across the paper at the origin. Carrier tetra- and tri-iodothyroacetic acid were developed with a sample of the extract at one side of the paper. The spots were separated to allow the solvent to pass more evenly up the paper. After development the carriers were stained by the method of Gross & Leblond (1951) and the ^{131}I-labelled compounds on the corresponding carrier-free parts of the chromatograms were eluted into methanol–aq. NH_3 soln. (sp.gr. 0·88) (3:1). The eluates were concentrated under reduced pressure below 40° and analysed by two-dimensional chromatography. Since the fatty material had a small R_F value in the two solvent systems used it was not present in these final eluates. The radioactive spots on the chromatograms were located by radioautography on Kodak No-screen X-ray film.

* This work forms part of a Ph.D. thesis submitted to the University of London.

Carriers. Thyroxine, tri-iodothyronine, tetra- and tri-iodothyroacetic acid, the corresponding iodothyropropionic acids and iodide were developed with the eluates on many chromatograms for identification purposes.

Control experiments. In order to check that no artifacts were produced from thyroid hormones present in the tissues, during extraction and analysis, experiments were carried out in which thyroxine or tri-iodothyronine biosynthetically labelled with ^{131}I was added to normal liver and kidney before homogenization. The tissues were subsequently extracted and analysed as described above.

RESULTS

Liver. Two-dimensional chromatographic analysis of the radioactive eluates obtained from the preliminary chromatograms, followed by radioautography, revealed that a number of iodinated compounds were present (Fig. 1), some of which were identified with one or other of the carriers; tri-iodothyronine, iodide and thyroxine were always present (spots *A*, *B*, *D*), and a compound (spot *C*) which had the same R_F values as tri-iodothyroacetic acid in the two solvent systems was observed on chromatograms of extracts prepared from mice killed later than 18 hr. after ^{131}I administration. The maximum total liver radioactivity was reached between 18 and 24 hr.; compound *C* was not detected at 12 hr. when relatively little organic ^{131}I was present in the liver. Compound *C* was detected on chromatograms developed with and without carrier. It represented only 0·1 % of the total liver radioactivity; this value was obtained from preliminary chromatograms by locating the relevant area with carrier tri-iodothyroacetic acid; the value was only approximate since no correction could be made for any decomposition of the compound which might have occurred during extraction, or for additional radioactivity from other compounds incompletely separated from it during chromatography. No tetraiodothyroacetic acid was detected in the livers of any mice even after exposure of the chromatograms for 3 weeks.

Kidney. Tri-iodothyroacetic acid was never found in the kidney; as the total radioactivity was considerably less than that in liver from the same animal, the technique may not have been sensitive enough to detect traces of this compound. However, analysis of the ^{131}I eluted from the tetraiodothyroacetic acid area of the preliminary chromatograms revealed that this acid and tri-iodothyronine, only partially separated, were present (Fig. 2).

Serum. Serum from each group of mice was analysed chromatographically to determine whether any thyroglobulin was present in the blood, thereby indicating radiation damage to the thyroid. Thyroglobulin was only detected 22 hr. after injection of ^{131}I. The amount then increased rapidly and at 48 hr. after ^{131}I administration represented at least 60 % of total serum ^{131}I.

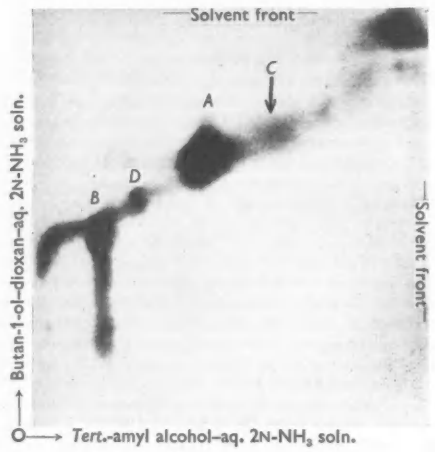


Fig. 1. Radioautograph of chromatogram of liver extract containing tri-iodothyroacetic acid. Solvent systems: butan-1-ol–dioxan–aq. 2N-NH_3 soln. and *tert.*-amyl alcohol–aq. 2N-NH_3 soln. *A*, Tri-iodothyronine; *B*, iodide; *C*, tri-iodothyroacetic acid; *D*, thyroxine.

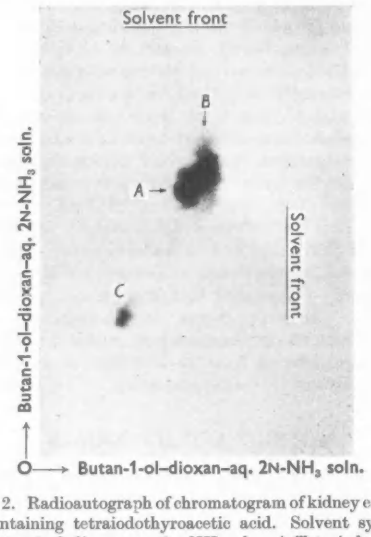


Fig. 2. Radioautograph of chromatogram of kidney extract containing tetraiodothyroacetic acid. Solvent system: butan-1-ol–dioxan–aq. 2N-NH_3 soln. *A*, Tetraiodothyroacetic acid; *B*, tri-iodothyronine; *C*, iodide.

Control experiments. No tetra- or tri-iodothyroacetic acid was detected in the kidney or liver to which ^{131}I-labelled thyroxine or tri-iodothyronine had been added before extraction.

DISCUSSION

Certain derivatives of thyroxine and tri-iodothyronine have been found to be inseparable by any analytical procedure so far examined. These include the corresponding thyroacetic acid and thyropropionic acid analogues. It may therefore be premature to identify tri-iodothyroacetic acid as a thyroid-hormone metabolite in biological material. However, certain facts support the hypothesis that the unknown metabolite found in the liver of mice after injection of ^{131}I is indeed this acid. When extracts were developed in the presence of carrier tri-iodothyroacetic acid a more diffuse spot of the ^{131}I-labelled compound was formed than that observed on carrier-free chromatograms, and the shape of the radioactive spot revealed by radioautography and that of carrier visualized by staining were very similar. These phenomena were not seen when carrier tri-iodothyropropionic acid was employed; it was sometimes found that although the radioactive spot and carrier spot overlapped, they could not be superimposed. Further, tri-iodothyroacetic acid might be produced from tri-iodothyronine in the tissues via the well-known biochemical pathway of oxidative deamination, whereas the propionic acid analogue cannot; reductive deamination of amino acids is not known to take place in biological tissues.

Since tetra- and tri-iodothyroacetic acid were not found to be artifacts formed during extraction or chromatography it was concluded that they represent metabolites of the thyroid hormones. Although the dose of ^{131}I used in this work was large and traumatic changes in the thyroid gland inevitably occurred, the thyroacetic acids were detected in the tissues before any thyroglobulin appeared in the blood.

Certain observations of Thibault and her colleagues support the suggestion that tetra- and tri-iodothyroacetic acid may be active thyroid-hormone metabolites. Gross, Pitt-Rivers & Thibault (1953) found that tri-iodothyronine, like thyroxine, when injected into rats stimulates the oxygen consumption only after a latent period. Thibault (Thibault & Pitt-Rivers, 1955; Thibault, 1956) observed that the corresponding thyroacetic acids produced an immediate increase in oxygen consumption both *in vivo* and in rat kidney slices *in vitro*. The fact that these derivatives have now been identified in certain tissues might lend support to this view. However, the immediate effects of tetra- and tri-iodothyroacetic acid have not been confirmed (Barker & Lewis, 1956; Dickens & Salmony, 1956; Wiswell & Asper, 1958), and it therefore cannot be said at present that the hypothesis has been justified by experimental evidence.

Although tetra- and tri-iodothyroacetic acid are formed in the tissues, this does not necessarily imply that they are biologically important; degradation of the thyroid hormones by deamination and decarboxylation in the tissues may represent only a minor process in thyroid-hormone metabolism.

Probably the most important metabolic pathway for the thyroid hormones is deiodination, and the large amount of work on this subject has been summarized by Tata *et al.* (1957), but it is not certain whether this results in the formation of partially iodinated compounds. Many organs and extravascular tissues of vertebrates possess some deiodinating activity; whether the deiodination of the thyroid hormones is directly related to their physiological activity has yet to be discovered.

SUMMARY

1. The acetic acid analogues of thyroxine and tri-iodothyronine have been identified in the kidneys and liver of mice given large doses of ^{131}I.

2. These compounds constitute approximately 0·1 % of the total radioactivity in the tissues.

REFERENCES

Barker, S. B. & Lewis, W. J. (1956). *Proc. Soc. exp. Biol., N.Y.*, **91**, 650.

Dickens, F. & Salmony, D. (1956). *Biochem. J.* **64**, 645.

Flock, E. V., Bollman, J. L., Grindlay, J. H. & McKenzie, B. F. (1957). *Endocrinology*, **61**, 461.

Gross, J. & Leblond, C. P. (1951). *Endocrinology*, **48**, 714.

Gross, J., Pitt-Rivers, R. & Thibault, O. (1953). *C.R. Soc. Biol. Paris*, **147**, 75.

Harington, C. R. & Pitt-Rivers, R. (1952). *Biochem. J.* **50**, 438.

Pitt-Rivers, R. (1953). *Lancet*, ii, 234.

Roche, J., Michel, R., Jouan, P. & Wolf, W. (1956). *Endocrinology*, **59**, 425.

Roche, J., Michel, R. & Tata, J. R. (1954). *Biochim. biophys. Acta*, **15**, 500.

Tata, J. R. (1957). *Proc. Soc. exp. Biol., N.Y.*, **95**, 362.

Tata, J. R., Rall, J. E. & Rawson, R. W. (1957). *Endocrinology*, **60**, 83.

Thibault, O. (1956). *Arch. Sci. physiol.* **10**, 423.

Thibault, O. & Pitt-Rivers, R. (1955). *Lancet*, i, 285.

Tomita, K., Lardy, H. A., Larson, F. C. & Albright, E. C. (1957). *J. biol. Chem.* **224**, 387.

Vogel, A. I. (1946). *Textbook of Organic Chemistry*, p. 175. London: Longmans, Green and Co.

Wiswell, J. G. & Asper, S. P. (1958). *Johns Hopk. Hosp. Bull.* **102**, 115.

The Biosynthesis of Polyacetylenes

2. ORIGIN OF THE CARBON ATOMS*

By J. D. BU'LOCK and H. GREGORY
Department of Chemistry, The University, Manchester

(*Received* 19 *November* 1958)

The principal acetylenic metabolites of the basidiomycete B. 841 (probably a *Poria* sp.) are known to be the hydroxy acids nemotinic acid (I) and odyssic acid (II), together with derivatives such as the corresponding lactones (Bu'Lock, Jones & Leeming, 1955, 1957). Acetylenic compounds of various kinds are now known to be relatively common constituents of certain groups of plants and fungi, and it is natural that interest should be felt in problems of their origin and their relationship to more conventional metabolites. In such a connexion the polyacetylenes from B. 841 form an apt group for further study, since they are produced fairly rapidly and in good yields (up to 1 mg./ml.) from culture media of simple constitution and, as all the compounds have strong absorption maxima at substantially the same wavelengths, their total amount is readily determined. The structures of (I) and (II) are not symmetrical so that isotopic studies are not likely to be vitiated by spurious randomization of labelling, a situation which would prevent comparable studies in many other polyacetylene-producing fungi; moreover, as is shown in this paper, the molecules of (I) and (II) are derived entirely from a single metabolic intermediate, a consideration which is bound to simplify the relationship between their synthesis and other metabolic processes in the fungus.

The gross characteristics of the formation of polyacetylenes from glucose in B. 841 have already been established (Bu'Lock & Leadbeater, 1956). It was shown that they are probably formed from an intermediate in normal glucose breakdown, and that the extent to which metabolized glucose carbon was diverted into polyacetylene synthesis was diminished during active growth of the fungus and could be increased by omitting essential growth factors from the nutrient medium. The present work is concerned with the identification of the intermediate from which the carbon atoms of (I) and (II) are derived.

METHODS

Culture of the fungus. Methods of growing the fungus B. 841 have already been described (Bu'Lock & Leadbeater, 1956). Experiments were carried out with cultures which had been supplied with the full growth medium for 6 weeks, followed by 4% glucose for 4 weeks, after which the medium was replaced with fresh 4% glucose containing the labelled substrate. The medium was drawn off for extraction after incubation for 7–9 days.

Extraction and purification of polyacetylenes. The culture medium was repeatedly extracted with ethyl acetate, and the polyacetylene content of the combined extracts estimated spectroscopically. The extract was then resolved by countercurrent distribution as described by Bu'Lock *et al.* (1955) and the nemotinic acid fractions were purified similarly. At the same time a more hydrophilic fraction was obtained, which contains (I) in bound form ('combined nemotinic acid') and this was purified by redistribution between ethyl acetate and water. With the radioactive polyacetylenes only those fractions showing a constant specific activity (per unit extraction) were combined, and the others discarded. The radioactive compounds were diluted, when necessary, with purified material.

Incorporation of acetate and succinate and oxidation of nemotinic acid to succinic acid

The labelled substrate (20 μc; 0·3–0·8 mg. in 20 ml. of sterile water) was added to a single culture flask; on working up, about 20 mg. of purified nemotinic acid (I) was obtained in each case. The (I) from [1-^{14}C]acetate was diluted with purified material, the degree of dilution being determined from the change in specific activity; the (I) from [1:4-$^{14}C_2$]succinate was not diluted. The (I) was counted as an 'infinitely thin' film of polymer (up to 0·6 mg. on 2·5 cm.^{2}) deposited on planchets by the evaporation of aqueous solutions under an infrared lamp, and the original acetate and succinate solutions were counted similarly. Weighings were carried out on a microbalance and it was found that for films of up to 1 mg. on 2·5 cm.^{2} no self-absorption correction was necessary.

Oxidation. In a typical experiment, a solution of (I) (47 mg.) in water (25 ml.) was mixed with 85 ml. of permanganate–periodate reagent (Rudloff, 1956) and sufficient K_2CO_3 to bring the solution to pH 7·7. The mixture was shaken for 6 hr., the excess of iodine removed with sodium metabisulphite, and the solution acidified to pH 3·0 with aq. 10% H_2SO_4 and continuously extracted with ether for 12 hr. The ether extract was evaporated to dryness, the residue neutralized with Na_2CO_3, reacidified with dilute HCl and evaporated to dryness. The crude succinic acid so produced was converted into the *p*-phenylazo-anil as follows (Henbest & Owen, 1955): the crude acid was heated with acetic anhydride (1 ml.) for 30 min. at 100° and the mixture evaporated to dryness under reduced pressure. The residual succinic anhydride, purified when necessary by vacuum sublimation, was dissolved in $CHCl_3$ (0·5 ml.) and heated under reflux with a solution of chromatographically

* Part 1: Bu'Lock & Leadbeater (1956).

pure *p*-aminoazobenzene in $CHCl_3$ (1 ml.) for 30 min. The anilic acid which separated on cooling was heated with acetyl chloride (2 ml.) until it dissolved; the solution was then evaporated under reduced pressure and the crystalline residue dissolved in benzene and purified by chromatography on Al_2O_3 to give succino-*p*-phenylazo-anil, m.p. 218° (yield 12–20% from nemotinic acid). A weighing pipette was used to deposit known amounts of the anil from toluene solution as 'infinitely thin' films for counting.

Incorporation of [1-^{14}C]*acetate and complete degradation of nemotinic acid*

Five B. 841 cultures were incubated for 9 days with a total of 80 μc (1–2 mg.) of [1-^{14}C]acetate. The nemotinic acid was isolated as above, counted as a thin film and then hydrogenated in ethanol with a platinum catalyst. The solution was filtered and evaporated, leaving an oil which on distillation (180°/15 mm. Hg) gave γ-undecanolactone in about 80% yield. Similarly, the 'combined nemotinic acid' was hydrogenated and the crude product treated with boiling ethanolic NaOH (5% in 95% ethanol) for 30 min. The solution was acidified and extracted with ether, and the ether extract evaporated and distilled to give a further quantity of the γ-undecanolactone.

A small sample of labelled γ-undecanolactone (90 mg.) was converted into γ-oxoundecanamide (Bu'Lock *et al.* 1955) and the purified product (m.p. 130°) counted by a thick-film technique for comparison with the $BaCO_3$ from subsequent degradations.

Alkali fusion of γ-undecanolactone. In a typical procedure the lactone (0·4 g.) was fused with powdered KOH (2·5 g.) in a nickel crucible contained in a Pyrex vessel, through which N_2 could be passed and which is heated in a bath of Wood's metal. The temperature was raised to about 350° over 10 min.; it was found that at approx. 250° no fission occurred, and that if the reaction was carried out in air or on a larger scale the final yields were lower. When the emerging N_2 was passed through a cold trap, no major quantity of volatile product was retained. The fusion mixture was kept at 350° until evolution of gas ceased (20–30 min.), then cooled, dissolved in water, washed twice with ether, acidified and subjected to steam-distillation. The fore-run, containing a little acetic acid, was discarded, and titration of the rest of the steam-distillate showed fission to two acids in about 56% yield. The titrated solution was acidified and extracted with ether, and the ether extract evaporated into water (40 ml.); the aqueous solution was fractionated in a six-tube countercurrent system between water and *n*-hexane. Tubes 1 and 2 contained propionic acid (identified by paper chromatography and as the *p*-phenylphenacyl ester) in 55% yield, tubes 3 and 4 were discarded and tubes 5 and 6 contained octanoic acid, similarly identified, in 54% yield. The acids were recovered by acidification and ether extraction. Vapour-phase chromatography of the methyl ester prepared from the octanoic acid fraction showed the presence of small amounts of heptanoic acid and nonanoic acid, which could be removed by reversed-phase chromatography by the method of Silk & Hahn (1954).

Degradation of propionic acid. The propionic acid was oxidized to give $BaCO_3$ from $C_{(1)}$ of (I) and acetic acid (Nahinsky & Ruben, 1941), and the latter degraded to two samples of $BaCO_3$ from $C_{(2)}$ and $C_{(3)}$ of (I) by way of acetone (Popják, French, Hunter & Martin, 1951).

Degradation of octanoic acid. A sample of the octanoic acid was freed from homologues and decarboxylated by the Schmidt reaction (Blömstrand, 1954), giving $BaCO_3$ from $C_{(4)}$ of (I). The remainder was converted by the method of Hunter & Popják (1951) into hexanoic acid and acetic acid, which were separated from each other and from unchanged octanoic acid by countercurrent distribution between *n*-hexane and water–ethanol (50:50, v/v) (50 tubes). The acetic acid was degraded as described above, giving $BaCO_3$ from $C_{(4)}$ (discarded; see below) and $C_{(5)}$ of (I). The hexanoic acid was degraded in the same way as the octanoic acid, giving butyric acid and acetic acid (separated by countercurrent distribution between $CHCl_3$ and water); the acetic acid afforded $BaCO_3$ from $C_{(6)}$ and $C_{(7)}$ of (I). Schmidt degradation of part of the butyric acid afforded $BaCO_3$ from $C_{(8)}$ of (I). The remainder of the butyric acid was oxidized (as for propionic acid), giving a single sample of $BaCO_3$ from $(C_{(8)}+C_{(9)})$ of (I), the activity of $C_{(9)}$ being obtained by difference; the other product of this reaction was acetic acid, degraded as above to give $BaCO_3$ from $C_{(10)}$ and $C_{(11)}$ of (I).

The $BaCO_3$ samples from the above degradations were counted as thick films (25 mg. on 1 cm.^{2}) in an end-window counter, for periods sufficient to give a counting error of 4% or less; the γ-oxoundecanamide was counted similarly. The actual counting rates were comparatively low (340 counts/min. for γ-oxoundecanamide, 54 counts/min. for $BaCO_3$ from $C_{(1)}$) so that no correction for counter-paralysis was made; the self-absorption of the γ-oxoundecanamide was assumed to be equivalent to that of $BaCO_3$. Attempts at van Slyke–Folch oxidations of the saturated fatty acid derivatives gave $BaCO_3$ not properly representative of the samples.

RESULTS

Incorporation of [1-^{14}C]*acetate.* The total incorporation of [1-^{14}C]acetate into polyacetylenes from 4% glucose replacement cultures was always between 15 and 20% (assuming that all the spectroscopically determined polyacetylenes have the same activity as the purified nemotinic acid). This is considerably greater than the yield of polyacetylenes from glucose metabolized under similar conditions (on a carbon-content basis, about 4%).

The figures in Table 1 show the relative activities of the individual carbon atoms of (I), derived from [1-^{14}C]acetate, and show that this activity is virtually confined to the odd-numbered atoms. Within the limits of our experimental errors these carbon atoms are uniformly labelled. We regard the apparent slight labelling of certain even-numbered carbon atoms as spurious for the following reasons. The alkali fusion of undecanolactone gives not only octanoic acid and propionic acid, but also small amounts of other acids. In determining the activity recorded in Table 1 for $C_{(4)}$ the octanoic acid was specially purified by chromatography before being subjected to decarboxylation, but for the other degradations we used acids which had not been thus purified. The presence of these

impurities in small amounts, decreasing with successive purification steps, accounts for the low activities recorded here for $C_{(2)}$ and $C_{(6)}$.

Incorporation of [1:4-$^{14}C_2$]*succinate.* Table 2 shows the relative incorporations of [1-^{14}C]acetate and [1:4-$^{14}C_2$]succinate, both into the whole molecule of (I) and also into the group, $C_{(1)}$–$C_{(4)}$, isolated from (I) by oxidation to succinic acid. Though succinate is nearly as effective as acetate in increasing the yield of polyacetylenes in glucose refloods (unpublished observations), it is clear that radioactivity from the succinate carboxyl group is only incorporated into (I) to a very minor extent. The resultant activity in (I) is distributed between $C_{(1)}$–$C_{(4)}$ and the remainder of the molecule in exactly the same (1:2) ratio as the radioactivity from the acetate carboxyl group. Since the conversion of succinate into acetate via oxaloacetate and pyruvate leads to the loss of both the carboxyl-group carbon atoms, we conclude that in B. 841 there also exists a minor pathway of succinate breakdown in which at least one carboxyl-group carbon atom is transformed into the acetate carboxyl group.

DISCUSSION

The results set out in Table 1 strongly suggest that the whole molecule of nemotinic acid (I) is derived from acetic acid and that its 11 carbon atoms are assembled by the head-to-tail linkage of six C_2 units. Though it was impracticable to carry out a similar degradation of the homologous odyssic acid (II), it seems probable that the terminal methyl group of (II) represents the twelfth carbon atom of these six C_2 units and that in the formation of (I) this atom is somehow eliminated.

$HC{\vdots}C{\cdot}C{\vdots}C{\cdot}CH{:}C{:}CH{\cdot}CH(OH){\cdot}CH_2{\cdot}CH_2{\cdot}CO_2H$

(I) Nemotinic acid

$CH_3{\cdot}C{\vdots}C{\cdot}C{\vdots}C{\cdot}CH{:}C{:}CH{\cdot}CH(OH){\cdot}CH_2{\cdot}CH_2{\cdot}CO_2H$

(II) Odyssic acid

$HC{\vdots}C{\cdot}C{\vdots}C{\cdot}CH{:}C{:}CH{\cdot}CH{\vdots}CH{\cdot}CH{:}CH{\cdot}CH_2{\cdot}CO_2H$

(III) Mycomycin

The formation of (I) and (II) thus presents close analogies to the biosynthesis of simple fatty acids, at any rate in the assembling of the carbon skeleton. Such a conclusion has seemed probable for some other naturally occurring acetylenic compounds on quite general grounds (e.g. in the various C_{18} straight-chain fatty acids with one or more triple bonds) and is probably of general validity. It follows that the enzymic reactions leading to the formation of triple bonds are such that in nature they are associated with the enzyme systems synthesizing straight-chain fatty acids, and that the acetylenic groups are formed, directly or indirectly, from the $-CO{\cdot}CH_2-$ groups produced in the repeated condensation of acetyl-coenzyme A. Further, when triple-bond-forming mechanisms are active it is apparent that they do not operate to the exclusion of the normal reactions of fatty acid synthesis, by which, for example, the greater part of the molecule of tariric (6-octadecynoic) acid is clearly formed. Bearing these points in mind some tentative conclusions about the biosynthesis of (I) and related compounds can be reached.

Though hydroxyacids are intermediates in fatty acid synthesis, the hydroxyl groups of (I) and (II) are not attached to originally oxygenated (i.e. carboxyl group-derived) carbon atoms, and are therefore secondary features, such as might well arise by the hydration of a double bond. The structure of mycomycin (III) (Celmer & Solomons, 1952) therefore presents a close analogy, since in this compound there are two double bonds in place of the one postulated in a precursor of (I); we

Table 1. *Incorporation of* [1-^{14}C]*acetate*

Activities are expressed as percentage mean activity of $BaCO_3$ from 'active' C atoms, counted as 'infinitely thick' samples to within ±4%.

Substance		Activity
γ-Oxoundecanamide (corrected to mol.wt. of $BaCO_3$)		630
$BaCO_3$ from	$C_{(1)}$	100
	$C_{(2)}$	5
	$C_{(3)}$	96
	$C_{(4)}$	1
	$C_{(5)}$	103
	$C_{(6)}$	8
	$C_{(7)}$	102
	$C_{(8)}$	0
	$C_{(9)}$	103
	$C_{(10)}$	0
	$C_{(11)}$	96

Table 2. *Incorporation of acetate and succinate*

Activities are expressed as counts/min./mg., counted as 'infinitely thin' films to within the indicated limits, adjusted to the mol.wt. of nemotinic acid. In each experiment, 20 μc of labelled substrate was used.

Substrate	[1-^{14}C]Acetate	[1:4-$^{14}C_2$]Succinate
Total incorporation into polyacetylenes (%)	15	0·3
Activity of nemotinic acid (I)	342±7	32±4
Activity of succinic acid from (I), as *p*-phenylazoanil	111±7	10±1

would suggest that the hydroxylation step is an essential part of the processes leading to the formation of the diyne-allene group. Compared with (I), mycomycin contains an extra C_2 unit, and its double bonds are so situated that they might well result from $\alpha\beta$–$\beta\gamma$ double-bond shifts of the kind catalysed by crotonase (cf. Lynen, 1954). Such features are not, of course, peculiar to polyacetylenes; a related interplay of reactions of hydration and dehydration is almost certainly involved in the biosynthesis of compounds like ricinoleic acid.

The presence of the allene groups in (I)–(III) raises the question whether these are normal intermediates in triple-bond formation. The naturally occurring allenes are very readily transformed into non-allenic acetylenes, but it is conceivable that the allenic groups might, at an earlier stage, arise by equally ready rearrangements of acetylenic groups, since the position of allene–acetylene equilibria is largely determined by the degree of conjugation with adjacent unsaturation. Consequently in (I)–(III) the allene groups may represent either incomplete conversion into, or secondary transformations of, acetylenic groups. Whatever the process by which $-CO\cdot CH_2-$ is transformed into $-C\vdots C-$, it probably involves 'activated' intermediates, since the reverse reaction is highly exothermic.

The relationship between the C_{11} compound (I) and its C_{12} homologue (II) requires some comment. In cultures of B. 841, (I) and its derivatives are greatly in excess of (II), yet it is implicit in our results that (I) is formed from a C_{12} compound containing a terminal methyl group, either (II) itself or one of its precursors. Several other polyacetylenes with odd numbers of carbon atoms are now known as fungal metabolites (personal communication from Professor E. R. H. Jones), and these, like (I)–(III), all contain a terminal ethynyl group $-C\vdots CH$. This suggests that the loss of the terminal methyl groups occurs after the introduction of unsaturation. The mechanism of the elimination seems likely to involve oxidation to a carboxyl group followed by loss of carbon dioxide; in this connexion the apparent interconvertibility, in *Polyporus anthracophilus*, of compounds with terminal $-CH_3$, $-CH_2\cdot OH$ and $-CO_2H$ groups (Bu'Lock, Jones & Turner, 1957) is perhaps significant.

SUMMARY

1. Nemotinic acid incorporating radioactivity from [1-^{14}C]acetate has been completely degraded to show the labelling pattern.

2. Nemotinic acid, with 11 carbon atoms, is formed from six molecules of an acetic acid derivative, linked head to tail, with elimination of the terminal methyl group, and odyssic acid is probably formed similarly without demethylation.

3. The natural acetylenes are formed within the context of the reactions of fatty acid synthesis, the triple bonds arising directly or indirectly from $-CO\cdot CH_2-$ groups. With the addition of the triple-bond-forming mechanism and the demethylation reaction, the reactions of fatty acid synthesis will account for most features of the structure of naturally occurring acetylene derivatives.

REFERENCES

Blömstrand, R. (1954). *Acta chem. scand.* **8**, 1487.
Bu'Lock, J. D., Jones, E. R. H. & Leeming, P. R. (1955). *J. chem. Soc.* p. 4270.
Bu'Lock, J. D., Jones, E. R. H. & Leeming, P. R. (1957). *J. chem. Soc.* p. 1097.
Bu'Lock, J. D., Jones, E. R. H. & Turner, W. B. (1957). *J. chem. Soc.* p. 1607.
Bu'Lock, J. D. & Leadbeater, E. F. (1956). *Biochem. J.* **62**, 476.
Celmer, W. D. & Solomons, I. A. (1952). *J. Amer. chem. Soc.* **74**, 1870, 2245.
Henbest, H. B. & Owen, T. C. (1955). *J. chem. Soc.* p. 2968.
Hunter, G. D. & Popják, G. (1951). *Biochem. J.* **50**, 163.
Lynen, F. (1954). *Nature, Lond.*, **174**, 962.
Nahinsky, P. & Ruben, S. (1941). *J. Amer. chem. Soc.* **63**, 2275.
Popják, G., French, T. H., Hunter, G. D. & Martin, A. J. P. (1951). *Biochem. J.* **48**, 612.
Rudloff, E. von, (1956). *Canad. J. Chem.* **34**, 1413.
Silk, M. H. & Hahn, H. H. (1954). *Biochem. J.* **56**, 406.

The Amide and Carboxyl Groups of Bacitracin A

By D. L. SWALLOW* and E. P. ABRAHAM
Sir William Dunn School of Pathology, University of Oxford

(*Received* 15 *December* 1958)

The polypeptide antibiotic, bacitracin A, has been assigned the provisional structure I, in which → represents a C–N bond (Weisiger, Hausmann & Craig, 1955*a*, *b*; Lockhart & Abraham, 1954; Lockhart, Abraham & Newton, 1955).

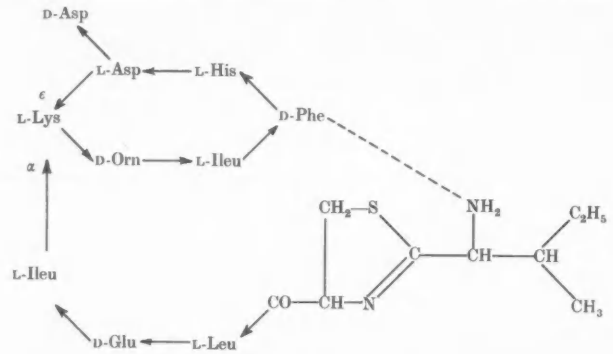
The precise nature of the linkages in several parts of this structure remained to be established. Bacitracin A contains two titratable acidic groups and yields one equivalent of ammonia on treatment with hot dilute acid. An amide group was therefore assumed to be present in the molecule, but its location was uncertain. Weisiger *et al.* (1955*a*) obtained a peptide from dinitrophenyl-bacitracin A which gave lysine and aspartic acid on hydrolysis but migrated as a base when subjected to electrophoresis on paper at pH 5·6, and they suggested that the aspartic acid in this peptide was present as an amide. However, Swallow & Abraham (1957, 1958) showed that a basic substance derived from the ε-aspartyl-lysine sequence in the molecule was not an amide but ε-(aminosuccinyl)lysine. It was not known whether L-aspartic acid was α- or β-linked to the ε-amino group of lysine and whether D-glutamic acid was α- or γ-linked to L-isoleucine. Moreover, the position of the D-aspartic acid residue had not been determined with certainty. The properties of certain peptides formed on partial hydrolysis of bacitracin A suggested that this residue projected, as shown, from a ring of six residues; but the alternative sequence

$$\text{L-His} \rightarrow \text{D-Asp} \rightarrow \text{L-Asp} \overset{\varepsilon}{\rightarrow} \text{L-Lys},$$

forming part of a ring of seven residues, was not rigidly excluded.

The present paper describes an attempt to obtain more information about these parts of the molecule from a study of the product obtained when esterified bacitracin A was reduced with lithium borohydride. Nystrom, Chaikin & Brown (1949) found that esters could be reduced to alcohols with lithium borohydride whereas amides were unaffected. The first attempt to apply the reaction to an esterified protein (insulin) gave promising results (Chibnall & Rees, 1951), but later studies indicated that the reaction might not have the desired specificity, since some reductive cleavage of peptide bonds could occur (Crawhall & Elliott, 1955). Soon after the present work had begun, Chibnall & Rees and their colleagues reported the results of a careful and detailed investigation into the use of lithium borohydride for the characterization of amide and *C*-terminal residues in proteins (Chibnall & Rees, 1958). They concluded that the reductive cleavage of peptide bonds was not a serious complication with proteins of relatively low molecular weight. Some of their procedures were closely followed in subsequent experiments with bacitracin.

* Present address: National Institute for Medical Research, The Ridgeway, Mill Hill, London, N.W. 7.



(I)

EXPERIMENTAL

Materials

Tetrahydrofuran. This was refluxed over K for 24 hr. and then distilled. It was stored over Na wire in the dark.

Lithium borohydride. The product from L. Light and Co. Ltd. was recrystallized shortly before use in the manner described by Chibnall & Rees (1958). The reducing power of this preparation was estimated by the iodate method of Lyttle, Jensen & Struck (1952).

Bacitracin. The bacitracin used was kindly supplied by the Commercial Solvents Corp., Terre Haute, Ind., U.S.A. (lot B55–13, 68 i.u./mg.). The main component of this material was bacitracin A, but some bacitracin B and other bacitracins were also present. Bacitracin A (100 i.u./mg.) was prepared from this material by the method of Newton & Abraham (1950), an 11-tube, 29-transfer, countercurrent distribution being used.

Amino alcohols and hydroxyamino acids. Samples of aspartidiol, γ-hydroxy-β-aminobutyric acid and δ-hydroxy-γ-aminovaleric acid were kindly provided by Dr A. C. Chibnall and Mr M. W. Rees. γ-Hydroxy-α-aminobutyric acid (homoserine) was obtained from the Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. δ-Hydroxy-α-aminovaleric acid was synthesized from γ-ethyl glutamate.

Synthesis of δ-hydroxy-α-aminovaleric acid. γ-Ethyl glutamate (0·59 g.), prepared by the method of King & Spensley (1950), and tetrahydrofuran (15 ml.) were placed in an apparatus similar to that described by Chibnall & Rees (1958). An eightfold molar excess of recrystallized $LiBH_4$ (0·52 g.) in tetrahydrofuran (20 ml.) was added, when the ester rapidly dissolved. The solution was heated (oil bath, 90°) so that it refluxed gently for 2 hr. It was then allowed to cool and a small excess of methanolic HCl added to decompose excess of $LiBH_4$ and any borane complexes. The product and inorganic material were precipitated by the addition of dry ether (100 ml.), the solid was washed twice with ether and finally freed from ether *in vacuo*. An aqueous solution of this product (0·5 g.) was desalted by electrophoresis in a four-compartment cell similar to that described by Synge (1951). When the current had fallen to a minimum of 20 mA at 500 V the contents of the specimen compartment were evaporated to dryness. The residue (0·36 g.) was recrystallized from a small volume of water. δ-Hydroxy-α-aminovaleric acid had m.p. 225° (decomp.) and ran with R_F 0·26 when subjected to chromatography on paper in butanol–acetic acid–water (Woiwod, 1949) (Found: C, 44·6; H, 8·2; N, 10·3. Calc. for $C_5H_{11}O_3N$: C, 45·1; H, 8·3; N, 10·5%).

δ-Hydroxy-α-aminovaleric acid was first prepared by Sörensen (1905) from the sodium derivative of phthalimidomalonic ester and αγ-dibromopropane.

Procedures

Esterification of bacitracin. A solution of bacitracin or bacitracin A in dry methanolic 0·1 N-HCl (1 g./100 ml.) was kept at 25° for 24 hr. in a stoppered flask (cf. Chibnall, Mangan & Rees, 1958*b*). The solvent was removed *in vacuo* in a rotary evaporator (Craig, Gregory & Hausmann, 1950), the residue was dissolved in a little dry methanol and reprecipitated by the addition of dry ether (100 ml.). The solid ester hydrochloride was separated by centrifuging, washed once with dry ether and freed from ether *in vacuo*.

Estimation of ammonia nitrogen in peptides. The amount of NH_3 nitrogen in bacitracin and bacitracin A was estimated by the method of Chibnall, Mangan & Rees (1958*a*). This method depends on the fact that a polypeptide may be precipitated from methanolic HCl by the addition of ether while ammonium salts remain in solution. The peptide (0·2 g.) was dissolved in methanolic 0·1 N-HCl (10 ml.) and immediately precipitated with ether (40 ml.). The precipitate was separated by centrifuging and the operation repeated twice. The final precipitate was washed with dry ether (50 ml.) at the centrifuge and freed from ether *in vacuo*. The recovery of peptide was 96%. The combined supernatants were evaporated to dryness and the residue was dissolved in water and introduced into a Markham still (Markham, 1942). After the addition of 4 ml. of M-Na_2HPO_4–NaOH buffer, pH 11·0, any NH_3 present was distilled over into 5 ml. of Conway reagent (Conway, 1935). The residual acid in the resulting solution was titrated with 0·01 N-$Ba(OH)_2$.

An essentially similar procedure was used to estimate the NH_3 nitrogen formed during esterification of bacitracin (100 mg.) in methanolic 0·1 N-HCl (25 ml.). In this case 150 ml. of ether was used for precipitation, since the ester hydrochloride was more soluble than bacitracin itself in ether–methanol. The recovery of ester hydrochloride was 85–90%.

Estimation of amide nitrogen in peptides. The peptides were first freed from NH_3 nitrogen by precipitation as described above. A sample of peptide (10 mg.) in 2 N-HCl (0·5 ml.) was heated in a sealed Pyrex tube at 100° for 3 hr. to liberate amide nitrogen as NH_4Cl. The tube was opened, two drops of bromocresol green solution added as an internal indicator and the solution adjusted to pH 5·2 with N-NaOH. The contents of the tube were then washed quantitatively into a Markham still and NH_3 was estimated as before.

Reduction of bacitracin ester hydrochloride with lithium borohydride. The sample to be reduced (about 100 mg.) was ground to a fine powder, weighed into a 50 ml. test tube and dried for 2 days *in vacuo* over P_2O_5. A 24-fold molar excess of $LiBH_4$ in tetrahydrofuran (10 ml.) was added and the tube fitted to a three-way adaptor carrying a mercury-sealed stirrer and a reflux condenser fitted with a $CaCl_2$ tube (cf. Chibnall & Rees, 1958). Stirring was begun and the reaction vessel placed in an oil bath at 90° so that the tetrahydrofuran refluxed gently. After 6 hr. the mixture was cooled and a small excess of methanolic HCl added, when the product dissolved. The solution was stirred for a further hour and dry ether (50 ml.) then added. The precipitate, consisting of reduced ester and inorganic material, was centrifuged, washed twice with dry ether and freed from ether *in vacuo*.

Hydrolysis of product of reduction. The product obtained on reduction of bacitracin ester with $LiBH_4$ was hydrolysed in 6 N-HCl at 105° for 24 hr., or in 12 N-HCl at 80° for 43 hr. (concn. about 20 mg./ml.). The resulting solutions were evaporated to dryness *in vacuo* in the presence of solid NaOH.

Desalting and preliminary separation of products of hydrolysis. (*a*) Neutral amino acids and hydroxyamino acids were separated from other products in a small amount of hydrolysate by electrophoresis on a sheet of Whatman no. 1 paper (14 v/cm. for 2·5 hr.) in collidine acetate buffer (0·05 M to acetate), pH 7·0. The apparatus

used was similar to that of Flynn & de Mayo (1951). About 2·5 mg. of hydrolysate was applied as a series of spots to the sheet (16 in. wide) and after electrophoresis the material showing no net charge at pH 7·0 was located and eluted with water as described by Lockhart & Abraham (1954).

(*b*) A larger amount (50 mg.) of hydrolysate was dissolved in about 2 ml. of water and added to a column (10 cm. × 0·9 cm. diam.) of Dowex-50 × 4 in the hydrogen form. Water (15 ml.) was passed through the column to remove inorganic anions. The resin was then stirred in a beaker with water (about 30 ml.) and aq. 2N-NH_3 soln. was added until the pH rose to about 9·5. The resin was separated by centrifuging and the supernatant fluid, containing mainly acidic and neutral amino acids and hydroxyamino acids, was evaporated *in vacuo*. The resin was again stirred with water (30 ml.) and 0·3N-$Ba(OH)_2$ added until the pH rose to about 11·5. The resulting eluate contained basic amino acids and other bases which were ninhydrin-positive. Ammonia was removed from the eluate by distillation *in vacuo*.

(*c*) The eluate obtained from Dowex-50 between pH 9·5 and 11·5 was added to a column (8·5 cm. × 0·9 cm.) of Dowex-2 × 8. The column had been first washed with 2N-NaOH until chloride-free and then with CO_2-free water until the percolate was neutral (cf. Rees, 1958). Bases with no acidic function passed through the column when the latter was washed with CO_2-free water and emerged in the first 14 ml. of percolate. Basic amino acids were subsequently eluted with N-HCl.

Examination of products by electrophoresis and chromatography on paper. Electrophoresis on Whatman no. 1 paper (14 v/cm. for 2·5 hr.) was carried out as described by Newton & Abraham (1954) in collidine acetate buffer (0·05M to acetate), pH 7·0, and in 10% (v/v) acetic acid, pH 2·3. Paper chromatograms were run on Whatman no. 1 paper in the following solvent systems: (*A*) butan-1-ol–acetic acid–water (4:1:4, by vol.) (Woiwod, 1949); (*B*) butan-1-ol–acetic acid–water (4:1:5, by vol.) (Partridge, 1948); (*C*) butan-2-one–propionic acid–water (15:5:6, by vol.) (Clayton & Strong, 1954); (*D*) triethylamine–acetone–water (1:16:3, by vol.) (Wright & Stadtman, 1956); (*E*) AnalaR phenol (75 g.)–water (21 ml.)–aq. NH_3 soln. (sp.gr. 0·88; 4·6 ml.) (Underwood & Rockland, 1954). Systems *A*, *B* and *E* were freshly prepared for each run. In system *C* the solvent began to drip from the end of the paper after 10 hr., but development of the chromatogram was continued for a total of 20 hr. After runs in systems *D* and *E* the papers were dried at room temperature in a stream of air. In general, 5–10 μg. of individual amino acids or hydroxyamino acids and 50–100 μg. of peptide hydrolysates were applied to the paper. Spots were revealed by ninhydrin.

RESULTS

Esterification of bacitracin

When subjected to electrophoresis on paper at pH 7·0, bacitracin A migrated as a single spot 2·2 cm. towards the cathode. (Under similar conditions glycine was carried 1·0 cm. towards the cathode by endosmosis.) The methyl ester of bacitracin A migrated 8·2 cm. towards the cathode at pH 7·0, and showed no evidence of contamination with smaller peptides or amino acids. The ultraviolet-absorption spectrum of the methyl ester hydrochloride of bacitracin A was very similar to that of bacitracin A itself (cf. Newton & Abraham, 1953*b*), indicating that the thiazoline ring had remained intact during the treatment with methanolic HCl.

The sample of commercial bacitracin used separated into two main components when subjected to electrophoresis on paper at pH 7·0. One component was indistinguishable from bacitracin A. The other behaved as though it had no net charge and was probably a mixture of bacitracin E and F (Newton & Abraham, 1953*a*; Craig, Weisiger, Hausmann & Harfenist, 1952). Esterification of commercial bacitracin yielded a product which also gave two main spots on electrophoresis at pH 7·0. One spot was found in the position occupied by the methyl ester of bacitracin A and one had migrated less far (5·5 cm.) towards the cathode.

The amounts of ammonia and amide nitrogen found in the different peptides are given in Table 1. This shows that about one-third of the amide nitrogen was removed during the course of esterification.

Identification of the products of reduction of aspartic acid and glutamic acid residues by chromatography and electrophoresis on paper

An aspartic acid residue that is α-linked in a peptide chain, or present as a *C*-terminal α-amide, should give γ-hydroxy-α-aminobutyric acid (II) on esterification and subsequent reduction with $LiBH_4$. Aspartic acid that is β-linked, or present

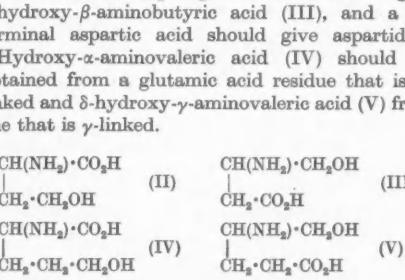
Table 1. *Ammonia and amide nitrogen in bacitracin and its methyl ester*

Estimations of amide nitrogen were done in triplicate on each sample. For methods of estimation see text.

Product	Equiv./1638 g. of product*	
	Ammonia N	Amide N
Bacitracin A hydrochloride	0·04	0·91
		0·88
		0·90
Bacitracin A methyl ester hydrochloride	0·28	0·59
		0·55
		0·64
Bacitracin	0·16	1·02
		1·08
		1·06
Bacitracin methyl ester hydrochloride	0·37	0·69
		0·68

* 1638 g. has been chosen as being close to the molecular weight of the hydrated bacitracin A hydrochloride isolated after countercurrent distribution (cf. Lockhart, Abraham & Newton, 1955). Provided that the methyl ester hydrochloride has the same degree of hydration, its molecular weight should not differ from this value by more than 3%.

as a *C*-terminal asparagine residue, should give γ-hydroxy-β-aminobutyric acid (III), and a *C*-terminal aspartic acid should give aspartidiol. δ-Hydroxy-α-aminovaleric acid (IV) should be obtained from a glutamic acid residue that is α-linked and δ-hydroxy-γ-aminovaleric acid (V) from one that is γ-linked.



The behaviour of the four hydroxyamino acids on paper chromatograms in different solvent systems (see Experimental section) is shown in Table 2. The compounds were separated into pairs in systems *A*, *B* and *E*, but the resolution of all four compounds was achieved only in system *C*. γ-Hydroxy-α-aminobutyric acid and δ-hydroxy-α-aminovaleric acid were not widely separated in system *C* but could readily be distinguished in system *D*. Distinction of the hydroxyamino acids from the neutral amino acids of bacitracin A presented no difficulty (Table 2).

Chibnall, Haselbach, Mangan & Rees (1958) found that γ-hydroxy-β-aminobutyric acid lactonized readily in acid solution. A sample of each of the four hydroxyamino acids was therefore heated at 100° in 6N-HCl for 1 min., the solution evaporated to dryness *in vacuo* and the residue tested for the presence of a lactone by electrophoresis on paper at pH 7. γ-Hydroxy-β-aminobutyric acid, γ-hydroxy-α-aminobutyric acid and δ-hydroxy-α-aminovaleric acid yielded spots which could be attributed to lactones (Table 2). The finding that the lactone of γ-hydroxy-α-aminobutyric acid migrated towards the cathode much more slowly than the lactone of γ-hydroxy-β-aminobutyric acid and somewhat more slowly than the lactone of δ-hydroxy-α-aminovaleric acid was not surprising, since the pK_a of the amino group (and thus its fractional positive charge at pH 7·0) should be lower the nearer the position of the amino group to the $-CO_2^-$ group. Under the conditions used here, about half of the γ-hydroxy-β-aminobutyric acid was converted into its lactone. The lactone from γ-hydroxy-α-aminobutyric acid was formed in smaller yield and appeared to be less stable. The lactone from δ-hydroxy-α-aminovaleric acid was detected only in trace amounts and appeared to undergo progressive hydrolysis during its migration at pH 7·0.

Products formed on reduction and hydrolysis of esterified bacitracin A

Esterification of bacitracin A and commercial bacitracin respectively, followed by reduction with $LiBH_4$ and hydrolysis, led to products which gave very similar patterns when subjected to electrophoresis or chromatography on paper. In their content of lysine, ornithine, histidine, leucine, isoleucine and phenylalanine these products resembled hydrolysates of bacitracin A itself, but they differed from the latter in containing no detectable amount of cystine. They also differed from hydrolysates of bacitracin A (and from hydrolysates of a sample of bacitracin A which had been treated with $LiBH_4$ without previous esterification) in several other respects.

A notable feature of the pattern obtained from hydrolysates of the reduced peptide ester on electrophoresis at pH 7·0 was the absence of a detectable spot in the position occupied by glutamic acid (Fig. 1). This was correlated with the presence, in the neutral fraction of the hydrolysate, of a substance which behaved as δ-hydroxy-α-aminovaleric acid on chromatography in systems *A*, *B*, *C* and *D*, and on electrophoresis at pH 2·3.

Table 2. *Chromatography and electrophoresis on paper of hydroxyamino acids and of neutral amino acids from bacitracin A*

For details of the solvent systems used in chromatography see text. Electrophoresis was for 2·5 hr. with a potential gradient of 14 v/cm.

Substance	R_F in system *A*	*B*	*D*	*E*	R_{Val} in system *C*	Migration (cm.) towards cathode pH 7·0	pH 2·3
γ-Hydroxy-α-aminobutyric acid (γ-α-HAB)	0·24	0·23	0·47	0·56	0·50	1·0	7·4
γ-Hydroxy-β-aminobutyric acid (γ-β-HAB)	0·31	0·28	0·41	0·60	0·71	1·0	15·0
δ-Hydroxy-α-aminovaleric acid (δ-α-HAV)	0·26	0·27	0·35	0·68	0·55	1·0	7·4
δ-Hydroxy-γ-aminovaleric acid (δ-γ-HAV)	0·36	0·35	0·43	0·70	0·96	1·0	15·0
γ-α-HAB lactone*	0·39	—	—	—	—	7·5	20·0
γ-β-HAB lactone†	0·32	0·33	—	—	1·09	13·0	—
δ-α-HAV lactone	—	—	—	—	—	9·0	—
Aspartidiol	0·36	0·35	0·57	—	>1·5	14·8	17·3
Leucine and isoleucine	0·67	0·61	0·48	—	1·32	1·0	7·2
Phenylalanine	0·63	0·56	0·48	—	1·32	1·0	6·6

* Grey colour with ninhydrin. † Yellow colour with ninhydrin.

When the reduced peptide ester was hydrolysed in 12N-HCl for 43 hr. at 80° it gave a product which was shown by electrophoresis at pH 7·0 to contain ε-(aminosuccinyl)lysine but only a trace of aspartic acid. When hydrolysis was carried out in 6N-HCl for 24 hr. at 105° the product contained about half as much aspartic acid as did a similar hydrolysate of bacitracin A (Fig. 1). Corresponding with the missing aspartic acid, a substance was present in the neutral fraction of the hydrolysate which behaved as γ-hydroxy-α-aminobutyric acid on chromatography in systems *A*, *B*, *C* and *D*, and on electrophoresis at pH 2·3. Treatment of the neutral fraction with 6N-HCl at 100° for 1 min. resulted in the formation of a substance which behaved as the lactone of γ-hydroxy-α-aminobutyric acid on electrophoresis at pH 7·0.

The neutral fraction of hydrolysates of reduced bacitracin A ester contained two further substances not found in hydrolysates of bacitracin A. One was indistinguishable from proline in systems *A*, *B*, *C* and *D*, and gave a bright-blue colour identical with that given by proline when the paper was sprayed with a solution of isatin in acetone (Smith, 1953). The other, which showed an R_F value similar to that of alanine in systems *A* and *B* but was distinguished from alanine by chromatography in system *C*, was not identified.

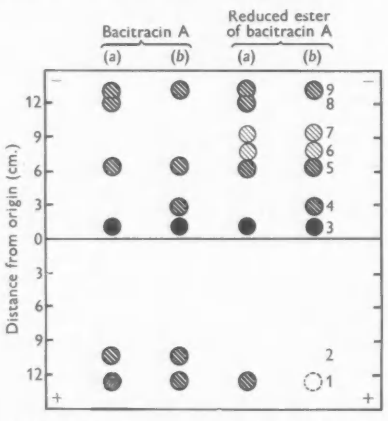


Fig. 1. Electrophoresis on paper at pH 7·0 (14 v/cm., 2·5 hr.) of hydrolysates of bacitracin A and the reduced ester of bacitracin A respectively. The depth of shading represents the approximate intensity of the spots revealed by ninhydrin. (*a*) Hydrolysis in 6N-HCl, at 105° for 24 hr.; (*b*) hydrolysis in 12N-HCl at 80° for 43 hr. 1, Aspartic acid; 2, glutamic acid; 3, neutral amino acids and hydroxyamino acids; 4, ε-(aminosuccinyl)lysine; 5, histidine; 6, γ-hydroxy-α-aminobutyric acid lactone; 7, δ-hydroxy-α-aminovaleric acid lactone; 8, lysine; 9, ornithine.

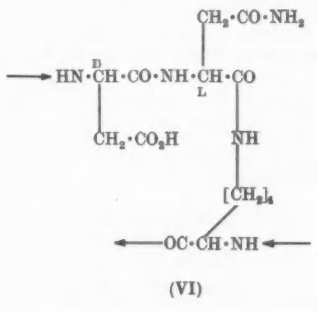
Small amounts of several new basic substances were revealed when hydrolysates of reduced bacitracin A ester were subjected to electrophoresis at pH 7·0. Two of these substances, which migrated less rapidly than lysine towards the cathode, corresponded to the lactones of γ-hydroxy-α-aminobutyric acid and δ-hydroxy-α-aminovaleric acid respectively (Fig. 1). The remaining substances, which migrated further than ornithine, could have consisted of some aspartidiol together with products of the reductive fission of the thiazoline ring and perhaps of peptide bonds. Basic substances with no acidic function were separated from other components of the hydrolysate by passage through Dowex-2. These substances, however, were obtained only in small yield and no definite conclusions about their identity could be reached after they had been examined by chromatography or electrophoresis on paper.

DISCUSSION

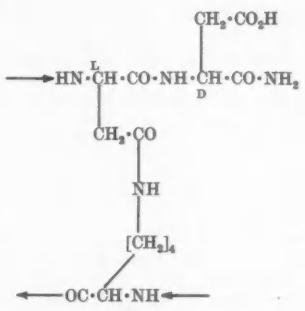
The absence of glutamic acid from hydrolysates of reduced bacitracin A ester indicates that the D-glutamic acid residue in bacitracin A does not itself carry the single amide group of the bacitracin A molecule, while the presence of δ-hydroxy-α-aminovaleric acid and small amounts of proline in these hydrolysates indicates that the glutamic acid residue is α-linked in the peptide chain and that its γ-carboxyl group is free. Proline was shown by Sörensen, Höyrup & Andersen (1911) to be formed from δ-hydroxy-α-aminovaleric acid in the presence of hot concentrated hydrochloric acid.

The presence of ε-(aminosuccinyl)lysine in the product obtained by hydrolysing reduced bacitracin A ester in 12N-hydrochloric acid at 80° indicates that neither of the two carboxyl groups of the L-aspartic acid residue in bacitracin A is free. One of these groups is involved in peptide linkage with the ε-amino group of lysine; the other could therefore be present as an amide or be joined to the residue of D-aspartic acid as shown in I and VII. In either case, one carboxyl group of the D-aspartic acid residue in bacitracin A would be free, and the finding that no significant amount of aspartic acid is liberated when the reduced ester is treated with 12N-hydrochloric acid at 80° would be understandable. The formation of γ-hydroxy-α-aminobutyric acid on hydrolysis of reduced bacitracin A ester thus leads to the conclusion that the β-carboxyl group of the D-aspartic acid residue in bacitracin A is free.

These findings can be accommodated by VI or VII, or by one of two corresponding structures in which the β- and α-carboxyl groups respectively of L-aspartic acid are linked to the ε-amino group of lysine. Thus in a structure of type VI the L-aspartic



(VI)



(VII)

acid would carry the amide group and could be either asparagine or *iso*asparagine. In a structure of type VII the D-aspartic acid would carry the amide group and be present as *iso*asparagine.

The esterification of bacitracin was accompanied by a significant loss of amide nitrogen and it therefore seemed likely that some of the amide group had been converted into an ester. Reduction of an ester grouping formed in this way and hydrolysis of the product should have yielded additional γ-hydroxy-α-aminobutyric acid from VI but some γ-hydroxy-β-aminobutyric acid from the corresponding structure in which the L-aspartic acid was β-linked to lysine. No γ-hydroxy-β-aminobutyric acid was in fact detected. With VII, or the corresponding structure in which the L-aspartic acid was α-linked to lysine, reduction of the new ester should have resulted in the formation of some aspartidiol. No clear evidence for or against the presence of significant amounts of aspartidiol in hydrolysates of the reduced peptide was obtained. Further information about this part of bacitracin A might be gained by hydrolysis of the amide group before esterification and reduction of the peptide, but it is not certain that the amide nitrogen can be removed without the occurrence of a transformation in the molecule (Craig, Konigsberg & Hill, 1958) and such a procedure would be unlikely to facilitate a decision between VII and the corresponding structure in which L-aspartic acid is α-linked to lysine.

The present work indicates therefore that the amide group in bacitracin A is associated with an aspartic acid residue and reduces the number of possible arrangements in this portion of the molecule from 18 to four, but it does not give positive support to any single structure of the four. It can be seen, however, that if either VI or VII was correct bacitracin A could be formed by two condensations in a linear dodecapeptide whose amino acids were linked by classical α-peptide bonds: condensation in an *N*-terminal isoleucylcysteinyl fragment could lead to the thiazoline ring and condensation of an aspartic acid carboxyl group with the ε-amino group of lysine could yield a cyclopeptide structure of six or seven residues.

SUMMARY

1. Bacitracin A and commercial bacitracin have been esterified by treatment with methanolic hydrogen chloride. About one-third of the amide nitrogen was removed during esterification.

2. The esterified peptides have been reduced with lithium borohydride and the products hydrolysed. The composition of the hydrolysates has been compared with that of similar hydrolysates of bacitracin A by chromatography and electrophoresis on paper.

3. Hydrolysates of the reduced peptide esters contained δ-hydroxy-α-aminovaleric acid and small amounts of proline, but no glutamic acid.

4. Hydrolysis of the reduced peptide esters with 12N-hydrochloric acid at 80° yielded ε-(aminosuccinyl)lysine but only traces of aspartic acid. Hydrolysis with 6N-hydrochloric acid at 105° yielded aspartic acid equivalent to one residue and γ-hydroxy-α-aminobutyric acid.

5. It is concluded from these results that the glutamic acid residue in bacitracin A is α-linked and that its γ-carboxyl group is free. It is also concluded that one of the aspartic acid residues in bacitracin A is present as an amide and that both carboxyl groups of the aspartic acid directly linked to the ε-amino group of lysine are bound. Alternative structures consistent with these conclusions are discussed.

One of us (D.L.S.) is indebted to the Medical Research Council for a scholarship.

REFERENCES

Chibnall, A. C., Haselbach, C., Mangan, J. L. & Rees, M. W. (1958). *Biochem. J.* **68**, 122.

Chibnall, A. C., Mangan, J. L. & Rees, M. W. (1958*a*). *Biochem. J.* **68**, 111.

Chibnall, A. C., Mangan, J. L. & Rees, M. W. (1958*b*). *Biochem. J.* **68**, 114.
Chibnall, A. C. & Rees, M. W. (1951). *Biochem. J.* **48**, xlvii.
Chibnall, A. C. & Rees, M. W. (1958). *Biochem. J.* **68**, 105.
Clayton, R. A. & Strong, F. M. (1954). *Analyt. Chem.* **26**, 1362.
Conway, E. J. (1935). *Biochem. J.* **29**, 2755.
Craig, L. C., Gregory, J. D. & Hausmann, W. (1950). *Analyt. Chem.* **22**, 1462.
Craig, L. C., Konigsberg, W. & Hill, R. J. (1958). In *Amino Acids and Peptides with Antimetabolic Activity*, p. 226. London: J. and A. Churchill Ltd.
Craig, L. C., Weisiger, J. R., Hausmann, W. & Harfenist, E. J. (1952). *J. biol. Chem.* **199**, 259.
Crawhall, J. C. & Elliott, D. F. (1955). *Biochem. J.* **61**, 264.
Flynn, F. V. & de Mayo, P. (1951). *Lancet*, **261**, 235.
King, F. E. & Spensley, P. C. (1950). *J. chem. Soc.* p. 3159.
Lockhart, I. M. & Abraham, E. P. (1954). *Biochem. J.* **58**, 633.
Lockhart, I. M., Abraham, E. P. & Newton, G. G. F. (1955). *Biochem. J.* **61**, 534.
Lyttle, D. A., Jensen, E. H. & Struck, W. A. (1952). *Analyt. Chem.* **24**, 1843.
Markham, R. (1942). *Biochem. J.* **36**, 790.
Newton, G. G. F. & Abraham, E. P. (1950). *Biochem. J.* **47**, 257.
Newton, G. G. F. & Abraham, E. P. (1953*a*). *Biochem. J.* **53**, 597.
Newton, G. G. F. & Abraham, E. P. (1953*b*). *Biochem. J.* **53**, 604.
Newton, G. G. F. & Abraham, E. P. (1954). *Biochem. J.* **58**, 103.
Nystrom, R. F., Chaikin, S. W. & Brown, W. G. (1949). *J. Amer. chem. Soc.* **71**, 3245.
Partridge, S. M. (1948). *Biochem. J.* **42**, 238.
Rees, M. W. (1958). *Biochem. J.* **68**, 118.
Smith, I. (1953). *Nature, Lond.*, **171**, 43.
Sörensen, S. P. L. (1905). *Bull. Soc. chim. Fr.*, **33**, 1052.
Sörensen, S. P. L., Höyrup, M. & Andersen, A. C. (1911). *Hoppe-Seyl. Z.* **76**, 44.
Swallow, D. L. & Abraham, E. P. (1957). *Biochem. J.* **65**, 39P.
Swallow, D. L. & Abraham, E. P. (1958). *Biochem. J.* **70**, 364.
Synge, R. L. M. (1951). *Biochem. J.* **49**, 642.
Underwood, J. C. & Rockland, L. B. (1954). *Analyt. Chem.* **26**, 1553.
Weisiger, J. R., Hausmann, W. & Craig, L. C. (1955*a*). *J. Amer. chem. Soc.* **77**, 731.
Weisiger, J. R., Hausmann, W. & Craig, L. C. (1955*b*). *J. Amer. chem. Soc.* **77**, 3123.
Woiwod, A. J. (1949). *J. gen. Microbiol.* **3**, 312.
Wright, B. E. & Stadtman, T. C. (1956). *J. biol. Chem.* **219**, 863.

Studies in the Biochemistry of Micro-organisms

106. METABOLITES OF *ALTERNARIA TENUIS* AUCT.: THE STRUCTURE OF TENUAZONIC ACID*

BY C. E. STICKINGS
Department of Biochemistry, London School of Hygiene and Tropical Medicine, University of London

(*Received* 16 *December* 1958)

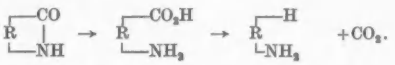
Rosett, Sankhala, Stickings, Taylor & Thomas (1957) described the isolation, from culture filtrates of *Alternaria tenuis* auct. grown on a glucose medium, of a number of metabolic products. Several of these appeared to be related to alternariol, a mycelial product formed by the same strains, and shown by Raistrick, Stickings & Thomas (1953) to be 3:4′:5-trihydroxy-6′-methyldibenzo-α-pyrone. The exception was tenuazonic acid, $C_{10}H_{15}O_3N$, the only nitrogen-containing metabolite among the new products; this was quite different in properties. This paper describes the work carried out on this acid and advances a structural formula. Rosett *et al.* (1957) described tenuazonic acid as an optically active viscous liquid, giving a strong orange–red ferric reaction and a green complex copper salt. It behaved as a monoketone and a monobasic acid, and was converted into an optical isomer on boiling with aqueous alkali, or on long keeping.

It has now been shown that hydrolysis of tenuazonic acid with 0·1N-mineral acid gives good yields of acetic acid and a colourless crystalline monobasic acid, m.p. 117·5–119·0°, which has the formula $C_8H_{13}O_2N$. This substance, shown below to be an epimeric mixture, will be called the deacetyl compound; it yields a mono-2:4-dinitrophenylhydrazone. In addition, tenuazonic acid gives iodoform with alkaline iodine. These reactions show the presence of both a $CH_3{\cdot}CO$ and a CO group in the molecule.

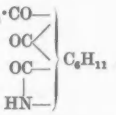
Hydrolysis of tenuazonic acid with 2N-mineral acid gives high yields of acetic acid, carbon dioxide and a basic compound, isolated as a benzenesulphonyl derivative, $C_7H_{14}ON{\cdot}SO_2C_6H_5$, m.p. 123·5–125·0°, which also has ketonic properties.

* Part 105: Birkinshaw & Chaplen (1958).

The benzenesulphonyl derivative is soluble in cold aqueous sodium hydroxide, hence the base is a primary amine; it is therefore an aminoheptanone; this substance is also produced in small amounts in the above-mentioned hydrolysis with 0·1 N-acid. The deacetyl compound, and tenuazonic acid itself, are both essentially non-basic. Together with the elimination of carbon dioxide, this suggests a lactam structure:



Tenuazonic acid can now be written

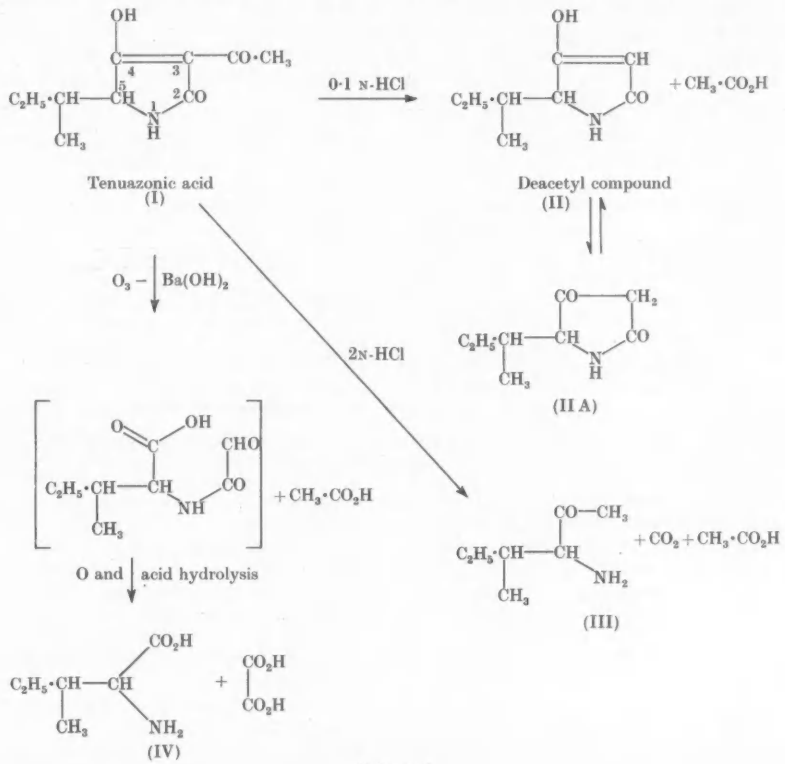


This accounts for all the oxygen and nitrogen atoms: the remainder of the molecule must consist of saturated hydrocarbon links and chains.

Ozonolysis of tenuazonic acid in aqueous alkali, followed by acid hydrolysis, yields acetic acid, oxalic acid and an amino acid. The latter is shown to be L-isoleucine by elementary analysis, optical rotation, paper-chromatographic behaviour and degradation by ninhydrin to a volatile aldehyde giving a 2:4-dinitrophenylhydrazone indistinguishable from (+)-2-methylbutanal 2:4-dinitrophenylhydrazone (cf. Fones, 1954).

Of the C_6 residue in the above partial formula for tenuazonic acid, five carbon atoms are accounted for by the branched hydrocarbon chain in isoleucine, and this chain must be attached to the nitrogen atom in tenuazonic acid. The base produced by strong acid hydrolysis must therefore be the methyl ketone corresponding to isoleucine, i.e. 3-amino-4-methylhexan-2-one (Scheme 1, III). This formulation is supported by the strong iodoform reaction given by the benzenesulphonyl derivative. The methyl in this $CH_3 \cdot CO$ group must arise from the other carbon atom in the C_6 residue.

The original acid is a strong monobasic acid (pK about 3·35), and the deacetyl compound is also a



Scheme 1

monobasic acid, though less strong. No carboxyl group is possible, hence there must be an enolic hydroxyl group in tenuazonic acid, i.e. a CH or CH_2 group α to at least two CO (or CO·NH) groups. This argument leads to the formula IIA or II for the deacetyl compound, and I for tenuazonic acid. Attachment of the acetyl group at position 3 would account for the stronger acidity of tenuazonic acid as compared with the deacetyl compound, the intense ferric reaction of tenuazonic acid and the formation of a complex copper salt.

Tenuazonic acid is thus α-acetyl-γ-*sec*-butyltetramic acid. This formulation explains most of the known facts. Thus ozonolysis in alkaline solution oxidizes the double bond, acetic acid is lost by hydrolysis and the terminal –CHO group is further oxidized to $-CO_2H$. Hydrolysis then yields oxalic acid and isoleucine (IV).

Hydrolysis under mild acid conditions leads to partial breakdown of the β-diketo-amide system, and with stronger acid the degradation is exactly comparable with that of α-acyltetronic acids under the same conditions (Clutterbuck, Raistrick & Reuter, 1935*a*, *b*). The chemistry of tenuazonic acid is in fact very similar to that of the α-acyltetronic acids, a number of which were isolated by Clutterbuck, Haworth, Raistrick, Smith & Stacey (1934) from strains of *Penicillium charlesii*. These

Table 1. *Ultraviolet-absorption maxima of tenuazonic acid, synthetic α-acetyltetramic acids, carlosic acid and α-acetyltetronic acid in various solvents*

Measurements were made in 1 cm. cells at concentrations of 15–20 mg./l.

	210–220 mμ		230–240 mμ		260–280 mμ	
Tenuazonic acid in	$\lambda_{max.}$	log ε	$\lambda_{max.}$	log ε	$\lambda_{max.}$	log ε
n-Hexane	214	3·69	—	—	274	4·06
Ethanol	217	3·72	—	—	277	4·13
Water	—	—	239	4·05	279	4·16
0·09 N-NaOH	—	—	240	4·07	279	4·17
0·09 N-HCl	220	3·80	—	—	277	4·10
α-Acetyl-γ-methyltetramic acid in ethanol*	—	—	240	3·72	280	4·04
α-Acetyltetramic acid in ethanol*	—	—	239	3·98	277	4·13
Carlosic acid† in						
Water	—	—	232	4·16	265	4·22
0·09 N-HCl	—	—	232	4·09	265	4·18
N-HCl	211	3·75	232	3·77	269	4·14
α-Acetyltetronic acid in						
0·025 N-H_2SO_4‡	—	—	230	4·18	265	4·18
0·5 N-HCl	211	3·85	230	3·95	261	4·07
2 N-HCl	211	3·94	—	—	260	4·01

* Lacey (1954).
† (−)-α-*n*-Butyryl-γ-carboxymethyltetronic acid (Clutterbuck, Raistrick & Reuter, 1935*b*).
‡ Herbert & Hirst (1935).

Table 2. *Main infrared-absorption maxima of tenuazonic acid and synthetic α-acetyltetramic acids in the regions* 3500–3000 *cm.*$^{-1}$ *and* 2000–1500 *cm.*$^{-1}$

Figures for α-acetyl-γ-methyltetramic acid and α-acetyltetramic acid are derived from curves (kindly supplied by Dr R. N. Lacey) which were recorded on a Hilger H. 800 double-beam instrument.

Tenuazonic acid		α-Acetyl-γ-methyl-tetramic acid	α-Acetyl-tetramic acid
Capillary layer (cm.$^{-1}$)	CCl_4 soln. (cm.$^{-1}$)	Nujol mull (cm.$^{-1}$)	Nujol mull (cm.$^{-1}$)
3296	3236	3380	3240
3112	3098	3074	3112
*	1735	1709	1729
*	1705 (shoulder)	—	—
*	1674	1680	1667
*	1630	1615	1620

* Absorption too strong for accurate measurement.

α-acyltetronic acids are strong acids, most of which yield similar ferric colours, copper derivatives and mono-dinitrophenylhydrazones.

Lacey (1954) has described the preparation of α-acetyltetramic acids by the reaction of diketen with amino acid esters, followed by ring closure. The properties of α-acetyltetramic acid, m.p. 155°, and α-acetyl-γ-methyltetramic acid, m.p. 115°, resemble those of tenuazonic acid, including a red ferric colour, strong acidity and stability of the ring system to boiling alkali. The absorption maxima are also similar (see Tables 1 and 2). In the ultraviolet region, the two synthetic compounds have absorption maxima at 239–240 mμ and 277–280 mμ in ethanol. Tenuazonic acid shows two maxima in absolute ethanol, at 217 and 277 mμ, and in *n*-hexane, at 214 and 274 mμ. However, in water there is no absorption peak at 217 mμ but there is one at 239 mμ. The spectrum in aqueous 0·09 N-sodium hydroxide is very similar to that in water, but in 0·09 N-hydrochloric acid the lower maximum is again shifted to 220 mμ. It would appear that the absorption at 239–240 mμ is due to an ionic form, whereas that at 214–220 mμ is due to a non-ionic structure. The shift to 220 mμ in 0·09 N-hydrochloric acid is surprising in view of the findings of Herbert & Hirst (1935), who showed that a number of α-acyltetronic acids absorbed at about 230 and 265 mμ in water, the maxima being essentially unchanged either in alkali or in 0·025 N-sulphuric acid. However, it has now been shown for carlosic acid and α-acetyltetronic acid that in stronger acid the intensity of the maximum at about 230 mμ is reduced and a new maximum appears at 211 mμ. The p*K* value of α-acetyltetronic acid is about 1·8 (Baker, Grice & Jansen, 1943); more concentrated acid is therefore required to suppress the ionization, than with tenuazonic acid. The infrared-absorption curves of the synthetic compounds are sufficiently close to that of tenuazonic acid to be consistent with its formulation as an α-acetyltetramic acid (Table 2).

Simple γ-alkyltetramic acids do not appear to have been described. Benary (1911) obtained tetramic acid, m.p. 211°, from α-carboxytetramic acid. It gave a blood-red colour with ferric chloride, and an intense violet with sodium nitrite (a reaction characteristic also of tetronic acids unsubstituted in the α-position). The deacetyl compound from tenuazonic acid reacts with sodium nitrite to give a purple–red but gives only a very slight brown ferric colour, though this deepens somewhat on standing. It is difficult to see why introduction of a *sec*-butyl group should reduce the ferric reaction so markedly. The deacetyl compound gives a slight positive iodoform reaction, despite the absence of a $CH_3 \cdot CO$ group. However, β-diketones are known to give a positive reaction in this test (Fuson & Tullock, 1934).

Although α-acyltetronic acids react smoothly with bromine (Clutterbuck *et al.* 1935*a*), bromination of α-acetyltetramic acids is complex (Lacey, 1954). The behaviour of tenuazonic acid itself towards bromine has not been studied, but the deacetyl compound reacts immediately with bromine water to give a high yield of a dibromo compound $C_8H_{11}O_2NBr_2$, which is insoluble in aqueous sodium bicarbonate but dissolves on treatment with sodium hydroxide. On acidification a new crystalline compound, $C_8H_{13}O_3NBr_2$, soluble in aqueous sodium bicarbonate, is precipitated. The structure of these dibromo compounds has not yet been elucidated. The dibromo derivative of (−)-γ-methyltetronic acid obtained by Clutterbuck *et al.* (1935*a*) also existed in a hydrated form.

The orientation about the two asymmetric carbon atoms in tenuazonic acid should be identical with the orientation about the corresponding atoms in the L-isoleucine obtained after ozonolysis and hydrolysis, since the acid hydrolysis of the amide link would not be expected to introduce any inversion. It is to be expected, however, that, while the asymmetric centre in the side chain would be stable, epimerization would take place readily at position 5 in the ring in both tenuazonic acid and its deacetyl derivative, owing to the α-carbonyl group. No doubt this is the explanation of the formation of the crystalline *iso*tenuazonic acid either on long keeping or, more rapidly, in boiling alkali (Rosett *et al.* 1957). It was therefore anticipated that ozonolysis of *iso*tenuazonic acid in alkali, followed by hydrolysis, under the same conditions as those used for tenuazonic acid, would yield D-*allo*isoleucine. In fact, the product obtained had practically zero specific rotation, but yielded the (+)-2-methylbutanal 2:4-dinitrophenylhydrazone in the same way as L-isoleucine: it was therefore a mixture of roughly equal parts of D-*allo*isoleucine and L-isoleucine.

It follows that the crystalline '*iso*tenuazonic acid' must be a mixture of diastereoisomers, which forms a crystalline copper salt of about the same relative composition. Accordingly, these two products have been re-examined, and by working up crystallization residues a copper salt of considerably higher dextrorotation has been obtained. However, this is probably not yet optically pure, and all attempts to separate the true *iso*tenuazonic acid or its salts or derivatives from tenuazonic acid by chromatographic methods have so far failed. The properties of the true *iso*tenuazonic acid and its derivatives are therefore unknown. The semicarbazone obtained from the crystalline acid may well be a mixture. It was recorded in the earlier paper that reaction with 2:4-dinitrophenylhydrazine yielded a mixture from which some tenuazonic acid 2:4-dinitrophenylhydrazone was isolated; it

was assumed at that time that epimerization had occurred during formation of the derivative, but this explanation is clearly no longer necessary.

The deacetyl compound is also a mixture. When ozonized in aqueous barium hydroxide and subjected to acid hydrolysis, it gave a mixture containing L-isoleucine and D-*allo*isoleucine in roughly equal quantities. The deacetyl compound therefore consists of approximately equal amounts of deacetyltenuazonic acid and deacetyl*iso*tenuazonic acid; the mixture crystallizes like a pure compound and no attempt has been made to separate the diastereoisomers. The same mixture is obtained by dilute acid hydrolysis of the crystalline '*iso*-tenuazonic acid'.

The hydrolysis product, 3-amino-4-methylhexan-2-one (III), also contains this labile asymmetric centre. The orientation of the particular isomer characterized as the benzenesulphonyl derivative, m.p. 123·5–125·0°, is unknown and it may also be a mixture; there are clear indications of the presence of two isomers in the reaction product.

So far as is known, tenuazonic acid is the first example of a substituted tetramic acid as a natural product. The tetronic acid derivatives produced by *P. charlesii* and other moulds (Clutterbuck *et al.* 1934, 1935*a*, *b*; Clutterbuck, Raistrick & Reuter, 1935*c*; Birkinshaw & Raistrick, 1936; Bracken & Raistrick, 1947) form the closest analogy, although the substituents at both 3- and 5-positions in tenuazonic acid are different from those in any of the natural tetronic acids so far described.

The ready breakdown to L-isoleucine, the naturally occurring form of this amino acid, suggests that tenuazonic acid is biosynthesized from L-isoleucine and 2 molecules of acetate, by elimination of 2 molecules of water. This follows closely the laboratory synthesis of α-acetyltetramic acids by Lacey (1954) mentioned above. Preliminary results indicate that tenuazonic acid can be synthesized in the laboratory in this way. The mode of biosynthesis of isoleucine is not fully known, but it is not directly formed from acetate. Ehrensvärd (1958) has evidence that, in two of the acyltetronic acids from *P. charlesii*, the α-side chain and carbon atoms 2 and 3 are similarly derived from acetate, whereas the remainder of the molecule arises by a different route.

EXPERIMENTAL

All melting points are corrected, except where otherwise stated. Microanalyses were by Weiler and Strauss (Oxford), Schoeller (Kronach, Germany) and Mr F. H. Oliver (Parke, Davis and Co. Ltd., Hounslow, Middlesex). Infrared spectra were determined by Miss E. M. Tanner of Parke, Davis and Co. Ltd. (except where otherwise stated), on a Grubb–Parsons double-beam instrument. Ultraviolet spectra were determined on a Hilger and Watts Uvispek spectrophotometer.

Tenuazonic acid

The following properties are in addition to those already described (Rosett *et al.* 1957).

Iodoform reaction. Tenuazonic acid (a few milligrams) was treated with 10N-NaOH, and just sufficient water was added to give a clear solution. Dropwise addition of a 10% solution of iodine in aq. 20% KI gave an immediate yellow precipitate and the characteristic smell of iodoform.

Sodium nitrite test. Tenuazonic acid was triturated with aq. $NaNO_2$. It dissolved to a yellow solution, but no red or violet developed even on long standing.

Approximate p*K value.* Titration of tenuazonic acid (0·196 g.) in methanol (10 ml.) and water (20 ml.) required 9·96 ml. of 0·1N-NaOH (Calc. for $C_{10}H_{15}O_3N$: 9·95 ml.). pH at half-neutralization (4·98 ml.) was 3·35.

Hydrolysis of tenuazonic acid by 2N-hydrochloric acid and formation of 3-amino-4-methylhexan-2-one

A mixture of tenuazonic acid (0·205 g.) and 2N-HCl (50 ml.) was boiled for 5·5 hr. in a stream of N_2. Water slowly distilled and was condensed, and boiled water was added at the same rate to the reaction flask. The stream of N_2 was carried through a bubbler containing Brady's reagent (0·32% of 2:4-dinitrophenylhydrazine in 2N-HCl), then through bubblers containing 0·2N-$Ba(OH)_2$, which were changed at intervals and titrated with 0·1N-HCl. Most of the CO_2 was evolved in the first 2 hr. [Found, after 5·5 hr.: 25·1 ml. of 0·1N-HCl. Calc. for 1 mol. of CO_2: 20·8 ml. The high result may be due to HCl spray (see below)]. The distillate was titrated at 50 ml. intervals; after 2·5 hr., the 50 ml. portions gave a fairly constant mean titre of 1·35 ml. of 0·1N-NaOH, and gave a positive chloride test. The titrated distillate was therefore treated with Ag_2SO_4 (0·5 g. in 100 ml. of water), filtered, treated with 2N-H_2SO_4 (1 ml.) and re-evaporated on the water pump for volatile-acid determination [Found: 8·95 ml. of 0·1N-NaOH (negative test for sulphate). Calc. for 1 equiv.: 10·4 ml.]. Only a slight precipitate appeared in the Brady's reagent.

The reaction solution was evaporated to dryness in a warm-water bath on the water pump. A bubbler containing Brady's reagent was interposed between receiver and pump, but no precipitate developed in it. The residual gum (0·16 g.) would not crystallize readily. A portion (0·14 g.) was dissolved in 2N-NaOH (6 ml.) and treated dropwise with benzenesulphonyl chloride (0·5 ml.) with shaking. The clear solution was acidified, cooled and filtered to give the crude benzenesulphonyl derivative, m.p. 84–110° (0·193 g.; 79% yield). Recrystallization from 75% methanol and cooling for a short time at room temperature yielded a fraction of m.p. 122–123° (0·07 g.); after further recrystallizations, this *benzenesulphonyl derivative of 3-amino-4-methylhexan-2-one* separated as colourless prisms, m.p. 123·5–125·0°; $[\alpha]_{5461}^{22} - 99 \pm 2°$ in methanol (*c*, 1·0) (Found: C, 57·95; H, 7·1; N, 5·1; S, 11·6. $C_{13}H_{19}O_3NS$ requires C, 57·9; H, 7·1; N, 5·2; S, 11·9%). The substance is soluble in cold 2N-NaOH. If this solution is treated with excess of Brady's reagent, yellow needles are produced. The iodoform test, carried out as described above, gives a strong positive result. Attempts to purify the residues from the

crystallizations were unsuccessful. Various fractions of m.p. between 84° and 90° were obtained, but did not recrystallize to constant m.p. One fraction, m.p. 84–85°, gave $[\alpha]_{5461}^{22} - 18 \pm 2°$ in methanol (*c*, 0·67) (see also below).

Identification of volatile acid as acetic acid. The titration solution was converted into the *p*-bromophenacyl derivative in the usual way. The crude derivative (m.p. 81–84°, 63% yield) was recrystallized from light petroleum (b.p. 60–80°). The crystals, m.p. 83·5–85·5°, were sublimed at 60–70° *in vacuo* to give a colourless sublimate, m.p. 85·0–86·5° alone or mixed with authentic *p*-bromophenacyl acetate; in each case the melt reset on cooling, and remelted at the same temperature (for an analysis, see next section).

Hydrolysis of tenuazonic acid and crystalline 'iso-tenuazonic acid' by 0·1 N-hydrochloric acid and formation of deacetyl compound

Tenuazonic acid (0·41 g.) and 0·1 N-H_2SO_4 (50 ml.) were boiled under reflux for 4·25 hr. in a stream of N_2; the effluent gas was passed through Brady's reagent, then through $Ba(OH)_2$, as described above. Carbon dioxide was evolved slowly throughout the experiment (Found: 4·40 ml. of 0·1 N-HCl. Calc. for 1 mol. of CO_2: 41·6 ml.). The precipitate in the Brady's reagent was negligible. Part of the hydrolysis solution was evaporated on the water pump for determination of volatile acid (Found: 7·9 ml. of 0·1 N-NaOH. Calc. for 1 equiv.: 8·4 ml.). Extraction of the remaining solution with ether or ethyl acetate yielded a largely crystalline product, which after washing with ether melted at 105–120°. After one recrystallization from ethyl acetate the *deacetyl compound* separated as colourless prisms, m.p. 117·5–119·0°, unchanged on further recrystallization; $[\alpha]_{5461}^{20} + 16 \pm 1°$ in methanol (*c*, 1·00) [Found: C, 62·2; H, 8·4; N, 8·8; equiv. by titration (phenolphthalein), 160. $C_8H_{13}O_2N$ requires C, 61·9; H, 8·4; N, 9·0%; mol.wt., 155].

In a second experiment, tenuazonic acid (2·12 g.) and 0·1 N-HCl (50 ml.) were refluxed for 4·75 hr. and the solution was extracted as described above. The ether washings were shown to consist largely of a mixture of starting material and *iso*tenuazonic acid (0·23 g.), and gave more deacetyl compound after further hydrolysis. The evolution of CO_2 suggested that some 3-amino-4-methylhexan-2-one was being formed. A portion of the extracted reaction solution was therefore treated with 2 N-NaOH and benzenesulphonyl chloride as described above. The yield of crude benzenesulphonyl derivative was 9%, m.p. 80–85°; $[\alpha]_{5461}^{22} - 23°$ in methanol (*c*, 1·00). This material (0·16 g.) was recrystallized from 75% methanol, yielding colourless prisms (0·03 g.), m.p. 121–124°; $[\alpha]_{5461}^{21} - 97 \pm 2°$ in methanol (*c*, 1·00). From the filtrate were obtained colourless needles (0·105 g.), m.p. 83–95°; $[\alpha]_{5461}^{22}$ 0 ± 1° in methanol (*c*, 1·00). Thus 11% of the starting material was not hydrolysed and another 9% was converted into the amine. The yield of crude deacetyl compound (0·83 g.) represents a further 50%; the purified material, m.p. 118–120°, weighed 0·68 g.; various crops of lower m.p. were obtained, and, in view of the results of ozonolysis described below, may contain the two isomers in different proportions.

The deacetyl compound gives only a slight brown with $FeCl_3$ in aqueous or ethanolic solution. On standing for some days this slowly intensifies to a moderate brown. The compound is soluble in water and methanol, but scarcely soluble in light petroleum and ether. An aqueous solution treated with Brady's reagent soon clouds and deposits yellow needles. If the deacetyl compound is dissolved in cold aq. $NaNO_2$, a pink colour develops after a few minutes, deepening to a purple–red on standing. It gives a positive, but not strong, iodoform reaction.

Identification of volatile acid. The titration solutions were evaporated to dryness and converted into the *p*-bromophenacyl derivative as described above; m.p. and mixed m.p. with *p*-bromophenacyl acetate, 86–87° (Found: C, 46·7; H, 3·5; Br, 31·15. Calc. for $C_{10}H_9O_3Br$: C, 46·7; H, 3·5; Br, 31·1%).

Hydrolysis of crystalline 'iso-tenuazonic acid'. The crystalline mixture gave essentially the same results as pure tenuazonic acid. After 3·5 hr., 9·5% of 1 mol. of CO_2 had been evolved. Evaporation yielded 79% of 1 equiv. of volatile acid, identified as acetic acid in the manner already described. The yield of recrystallized deacetyl compound was 34%, m.p. 117·5–119·0°, unchanged on mixing with the material from tenuazonic acid; $[\alpha]_{5461}^{21} + 15·5 \pm 1°$ in methanol (*c*, 1·00).

2:4-*Dinitrophenylhydrazone of deacetyl compound.* The deacetyl compound (21 mg.) was dissolved in ethanol (1 ml.) and Brady's reagent (15 ml.) was added. After standing overnight, the yellow crystals were collected (43 mg., m.p. 203·5–205·5°) and recrystallized from ethanol. The 2:4-*dinitrophenylhydrazone of the deacetyl compound* separated as fine yellow needles, m.p. 203·5–204·5° (Found, on material dried at 100° *in vacuo*: C, 50·4; H, 5·2; N, 20·4. $C_{14}H_{17}O_5N_5$ requires C, 50·2; H, 5·1; N, 20·9%). A dilute ethanolic solution gives a red with a drop of 2 N-NaOH.

Ozonolysis of tenuazonic acid and formation of L-isoleucine

Preliminary experiments showed that tenuazonic acid was rapidly ozonized in $CHCl_3$ solution, no ferric reaction being detectable after 25 min. The product, on boiling with 2 N-HCl, yielded a mixture containing oily volatile ketonic material, ammonium salts (detected by the Nessler test) and an amino acid which behaved chromatographically like DL-isoleucine. Ozonolysis in 0·1 N-NaOH was complete in 1 hr. and appeared to be less complex. Rough quantitative tests indicated that 25% of the N was converted into NH_3 and 30% into the amino acid.

Tenuazonic acid (0·79 g.) was dissolved in cold 0·16 N-$Ba(OH)_2$ (100 ml.) and ozonized O_2 was passed through for 2 hr. Some white solid was deposited (probably $BaCO_3$—see below). To this suspension was added a volume of 2 N-H_2SO_4 equivalent to 100 ml. of 0·16 N-$Ba(OH)_2$, and the mixture was then evaporated to dryness at 16 mm. Hg in a warm-water bath; two further small volumes of water were then added and distilled. The distillates were titrated with 0·1 N-NaOH (Found: 30·4 ml. of 0·1 N-NaOH. Calc. for 1 equiv.: 40·0 ml.). A quantity of 2 N-H_2SO_4 equivalent to 200 ml. of 0·16 N-$Ba(OH)_2$ was then added to the residue, and the solution was heated for 8·5 hr. on a steam bath. After cooling, the solution was extracted continuously for 10 hr. with ether. Evaporation of the ether left a mainly crystalline residue (0·54 g.) consisting largely of oxalic acid (see below). The reaction solution was now treated with 200 ml. of 0·16 N-$Ba(OH)_2$. A small portion was filtered and tested for Ba^{2+} and SO_4^{2-} ions in the usual way, and slight adjustments were made until both reactions were negligible. Finally, the $BaSO_4$ was removed by filtration

through fluted Whatman no. 50 paper, and the filtrate evaporated to dryness. The mainly solid residue (0·40 g.) was heated with absolute ethanol (4 ml.), most of the solid remaining undissolved. The mixture was cooled to 0–5° and filtered, leaving a nearly colourless solid (0·255 g.), which was sublimed at 170–180° in high vacuum on to a cold finger. [It has been shown by Gross & Grodsky (1955) that optically active amino acids, including L-isoleucine, sublime quantitatively and rapidly under these conditions with no racemization.] The colourless sublimate (0·22 g.; 42% of the theoretical yield of isoleucine) was crystallized twice from 80% ethanol, from which it separated as colourless plates with a silvery sheen, m.p. 284–285° (decomp.; sealed tube); $[\alpha]_D^{23}+14\pm1°$, $[\alpha]_{5461}^{23}+16\pm1°$ in water (*c*, 1·00); $[\alpha]_D^{22}+54\pm1°$, $[\alpha]_{5461}^{22}+63\pm1°$ in acetic acid (*c*, 1·98) (Found: C, 54·5; H, 9·9; N, 11·0. Calc. for $C_6H_{13}O_2N$: C, 54·9; H, 10·0; N, 10·7%). A specimen of L-isoleucine (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) had $[\alpha]_D^{23}+13\pm1°$, $[\alpha]_{5461}^{23}+14\pm1°$ in water (*c*, 1·02); $[\alpha]_D^{23}+53\pm1°$, $[\alpha]_{5461}^{23}+63\pm1°$ in acetic acid (*c*, 1·99). Until recently, there has been no satisfactory method of separating the four isomers of isoleucine, but the work of Greenstein, Levintow, Baker & White (1951) and Greenstein, Birnbaum & Otey (1953), based on specific enzymic hydrolysis of acetyl derivatives, has shown that L-isoleucine has $[\alpha]_D^{25}+13·5°$ in water (*c*, 1–5), +49° in acetic acid (*c*, 0·5–2·0); whereas for L-*allo*isoleucine, $[\alpha]_D^{25}$ is +18° in water (*c*, 1–5), +42·5° in acetic acid (*c*, 0·5–2·0). Using the Moore & Stein method (1951) of separation of amino acid mixtures, van Dam-Bakker (1958) showed that *allo*isoleucine could be separated from isoleucine, and that certain commercial preparations of isoleucine contained large amounts of *allo*isoleucine; however, she found that the L-isoleucine supplied by Nutritional Biochemicals Corp. was free from the *allo* isomer.

In another experiment, the rotation of the sublimate was determined before crystallization from aq. ethanol. The figures obtained {$[\alpha]_D^{20}+13\pm1°$ in water (*c*, 1·005), $+52\pm2°$ in acetic acid (*c*, 2·00)} indicate that the amino acid is optically homogeneous.

The amino acid was chromatographed at various stages of its purification in propan-1-ol–0·045M-sodium pyrophosphate buffer, pH 8·4 (4:1, v/v), on no. 1 Whatman paper previously soaked in the same buffer and dried (communication from Dr R. Thomas of this Department). After development, the paper was dipped in ninhydrin solution (0·1% in $CHCl_3$) and heated at 100°. In all cases, the major component was indistinguishable from DL-isoleucine, R_F 0·49–0·51 (descending). There was a slight impurity with R_F 0·35–0·37, and even smaller amounts with R_F 0·23 and 0·06. These were not completely eliminated by the sublimations and crystallization described.

Reaction of the amino acid with ninhydrin. Final proof of the structure of the amino acid was obtained by using the reaction with ninhydrin (Fones, 1954). The recrystallized material (40 mg.) was dissolved in water (15 ml.) and heated in a stream of steam. Ninhydrin (0·25 g. in 5 ml. of water) was added rapidly and the steam-distillation was continued until the distillate no longer clouded with Brady's reagent. Excess of Brady's reagent was added to the distillate and when precipitation was complete the yellow solid was filtered, washed and dried *in vacuo* over P_2O_5: wt. 56 mg., m.p. 131·0–132·5°; $[\alpha]_D^{22}+34\pm3°$ in $CHCl_3$ (*c*, 0·68). Recrystallization from ethanol yielded the (+)-2:4-dinitrophenylhydrazone of 2-methylbutanal as orange rectangular plates, m.p. 135–136°; $[\alpha]_D^{22}+33\pm3°$ in $CHCl_3$ (*c*, 0·74). One further crystallization raised the m.p. to 135·5–136·5°; $[\alpha]_D^{20}+30\pm2°$ in acetic acid (*c*, 1·00) (Found: C, 49·55; H, 5·3; N, 20·7. Calc. for $C_{11}H_{14}O_4N_4$: C, 49·6; H, 5·3; N, 21·0%). Fones (1954) gives m.p. 135–137°; $[\alpha]_D+30°$ in acetic acid (*c*, 1), +36° in $CHCl_3$ (*c*, 0·73). When the reaction was carried out with the sublimed amino acid, before recrystallization, the rotation was $[\alpha]_D^{22}+36\pm5°$ in $CHCl_3$ (*c*, 0·7).

L-Isoleucine (Nutritional Biochemicals Corp.) was subjected to the same reaction. The crude derivative had m.p. 129–132°; $[\alpha]_D^{23}+30\pm2°$ in $CHCl_3$ (*c*, 0·72). One recrystallization raised the m.p. to 135–136°; $[\alpha]_D^{23}+32\pm2°$ in $CHCl_3$ (*c*, 0·73). After one more recrystallization the derivative separated as orange plates, m.p. 135·5–136·5° either alone or mixed with the derivative obtained above; $[\alpha]_D^{21}+33\pm2°$ in $CHCl_3$ (*c*, 0·74), $+31\pm2°$ in acetic acid (*c*, 1·00).

Birkinshaw (1952) obtained this same 2:4-dinitrophenylhydrazone after ozonolysis of a hydrolysis product of sclerotiorin. A sample (kindly supplied by Professor J. H. Birkinshaw) did not depress the m.p. of the derivative described above.

The formation of an optically active aldehyde by this reaction eliminates the other possible structural isomers of formula $C_6H_{13}O_2N$. The specific rotation of the amino acid itself limits the choice to L-isoleucine and L-*allo*isoleucine, and the rotation of the above-mentioned 2:4-dinitrophenylhydrazone proves that the amino acid is L-isoleucine substantially unmixed with any other isomer.

Identification of volatile acid. The titration solution was converted into the *p*-bromophenacyl derivative as already described. The sublimed derivative melted at 85·5–86·5°, either alone or mixed with authentic *p*-bromophenacyl acetate.

Identification of non-volatile acid after hydrolysis. The mainly crystalline residue (0·54 g.) was treated with dry ether and filtered to give white prisms (0·25 g.; 50% of the theoretical yield), m.p. 100–102°; on slowly raising the temperature the melt reset, and remelted at 184–186° with vigorous effervescence and sublimation. A mixture with oxalic acid dihydrate (m.p. 100·5–102·0°, remelt 188–189°) melted at 100·5–102·0°, remelt 186–187°. A portion was titrated [Found: equivalent, 65·8. Calc. for $(CO_2H)_2.2H_2O$: 63·0]; the titration solution was then acidified with 2N-acetic acid and treated with excess of N-$CaCl_2$. The precipitated calcium salt was filtered and washed with water, dissolved in hot 2N-H_2SO_4, filtered and titrated with 0·1N-$KMnO_4$ [Found: equivalent, 63·5. Calc. for $(CO_2H)_2$, $2H_2O$: 63·0].

The ethereal filtrate from the crystalline oxalic acid was evaporated, dissolved in water and precipitated with $CaCl_2$. Titration of the calcium oxalate indicated that a further 13% of the theoretical yield of oxalic acid was present.

The identity of the oxalic acid was further confirmed by conversion of a portion into oxanilide, m.p. 255–257°, by boiling with aniline. Oxanilide prepared from authentic oxalic acid gave m.p. 256–258°, and a mixture melted at 256·0–257·5°. With an uncorrected thermometer the m.p. of oxanilide was 247–249°; the calculated correction for emergent stem was +8°. Most workers give the m.p. of this compound in the range 247–249° (e.g. Miller & Dittman, 1956; Roedig, Becker, Fugmann & Schoedel, 1955). The only higher figures found were 254° (Bornwater, 1912) and 252–253° (Figee, 1915). It is not stated whether these figures are corrected for emergent stem.

Ozonolysis of crystalline 'isotenuazonic acid'

'*iso*Tenuazonic acid', m.p. 60·0–62·5° (1·30 g.), was dissolved in 0·16 N-$Ba(OH)_2$ and ozonized and hydrolysed in the manner described above. The volatile acid was distilled (Found: 37·4 ml. of 0·1 N-NaOH. Calc. for 1 equiv.: 66·0 ml.). The non-volatile acid fraction (0·565 g.) gave white crystals (0·191 g.) identified as oxalic acid. The amino acid fraction, after washing with ethanol, weighed 0·53 g. (49% yield); $[\alpha]_D^{20}$ 0±1° in water (*c*, 0·99), unchanged on sublimation. The product was chromatographically indistinguishable from the product from tenuazonic acid. Reaction with ninhydrin followed by Brady's reagent, as already described, yielded a crude derivative, $[\alpha]_D^{23}$ +32±2° in $CHCl_3$ (*c*, 0·72). After two recrystallizations from ethanol, the compound melted at 135·5–136·0°; $[\alpha]_D^{23}$ +33±2° in $CHCl_3$ (*c*, 0·72). A mixture with the (+)-2:4-dinitrophenylhydrazone of 2-methylbutanal, obtained from authentic L-isoleucine, melted at 135·5–136·5°.

Ozonolysis of deacetyl compound

The deacetyl compound (155 mg., 1 m-mole) was dissolved in 0·189 N-$Ba(OH)_2$ (20 ml.) and ozonized O_2 was passed through for 2 hr. The solution was titrated with N-H_2SO_4 (with phenolphthalein): 1·42 ml. was required, indicating the presence of 2·36 equiv. of acid. N-H_2SO_4 (2·36 ml.) was now added, and the mixture was heated on a steam bath in a stream of N_2, the effluent being carried through $Ba(OH)_2$ bubblers for CO_2 estimation (Found: 0·57 ml. of N-H_2SO_4. Calc. for 1 mol. of CO_2: 2·00 ml.). Thus, apart from CO_2, 1·79 equiv. of acid was present (Calc. for oxalyl isoleucine, 2·00 equiv.). The solution was filtered and evaporated to dryness; further water was added and re-evaporated. Titration of the distillates gave, as expected, a negligible amount of volatile acid (5% of 1 equiv.). The residue was hydrolysed with N-H_2SO_4 and worked up as already described. The ether extract yielded a largely crystalline residue (130 mg.), which, when washed with ether, gave crystals of hydrated oxalic acid (60 mg.; 48% yield). The sublimed amino acid (52 mg.; 40% yield) behaved chromatographically as isoleucine; $[\alpha]_D^{23}$ −3±2° in water (*c*, 1·01).

Reaction with ninhydrin followed by Brady's reagent, as described above, yielded the crude derivative, m.p. 127–131°; $[\alpha]_D^{22}$ +32±5° in $CHCl_3$ (*c*, 0·74). After two recrystallizations from ethanol, the compound melted at 135–136°; $[\alpha]_D^{22}$ +32±2° in $CHCl_3$ (*c*, 0·73). A mixture with material derived from authentic L-isoleucine melted at 135·0–136·5°.

Bromination of deacetyl compound

The deacetyl compound (48 mg.) was dissolved in water (20 ml.) and treated dropwise with bromine water until a slight excess was present. The yellow colour was discharged with bisulphite and the crystals were filtered, m.p. 115·5–116·5° (70 mg., 72% yield). After recrystallization from water, then from light petroleum, the *dibromodeacetyl compound* separated as colourless needles, m.p. 116·5–117·5° (Found: C, 31·1; H, 3·7; Br, 50·7; N, 4·2. $C_5H_{11}O_2NBr_2$ requires C, 30·7; H, 3·5; Br, 51·1; N, 4·5%). The compound was insoluble in aq. $NaHCO_3$, but soluble in aq. NaOH. On acidification of the alkaline solution, well-formed prisms separated, m.p. 177·5–178·5°. Recrystallization from water and then aq. ethanol yielded glistening plates, m.p. 179–180° (Found: C, 29·1; H, 3·9; N, 4·9; Br, 47·9. $C_8H_{13}O_3NBr_2$ requires C, 29·0; H, 4·0; N, 4·2%; Br, 48·3). The compound is soluble in aq. $NaHCO_3$.

iso*Tenuazonic acid*

Copper tenuazonate trihydrate (0·76 g.) was converted into the free acid, and epimerized by boiling with 0·1 N-NaOH (59 ml.) for 9 hr. (see Rosett *et al.* 1957). It was then cooled and titrated to neutrality with 0·1 N-HCl, by the use of phenolphthalein. 0·1 N-Copper acetate (50 ml.) and $CHCl_3$ (50 ml.) were added, and the copper salt was extracted with further volumes of $CHCl_3$ until no further ferric colour was given by the extract. The $CHCl_3$ was evaporated and the residue dissolved in methanol (76 ml.). Measurement of rotation gave a minimum $[\alpha]_{5461}^{20}$ +56° in methanol (*c*, 1) [cf. copper salt of '*iso*tenuazonic acid' (trihydrate), $[\alpha]_{5461}^{22}$ +24·5° (Rosett *et al.* 1957)]. The solution was re-evaporated to dryness and immediately dissolved in $CHCl_3$ (7·6 ml.). After cooling at 0–5°, the crystals were filtered, washed and dried in air (0·58 g., equivalent to 0·43 g. of anhydrous); $[\alpha]_{5461}^{20}$ +30±1° in methanol (*c*, 1·00) for material dried at 100°. The filtrate was evaporated to dryness (0·27 g., equivalent to 0·25 g. of anhydrous); $[\alpha]_{5461}^{20}$ +116±2° in methanol (*c*, 1·00) for material dried at 100°. This residue was recrystallized from aq. methanol, yielding material of $[\alpha]_{5461}^{20}$ +134°, but further crystallization from this solvent did not increase the rotation significantly.

In another experiment, copper tenuazonate trihydrate (1·30 g.) and 2 N-NaOH (100 ml.) were heated to boiling for 1 min., cooled and filtered to remove cupric oxide. The rotation (α_{5461}^{20} +0·76°) was unchanged on boiling for a further 1·5 hr. The mixture was converted into the copper salt as above and crystallized from $CHCl_3$. The filtrate from the crystals was evaporated (0·35 g.), dissolved in warm $CHCl_3$ (3·5 ml.) and treated with benzene (14 ml.). The solid which separated on cooling was amorphous, but gave $[\alpha]_{5461}^{20}$ +176±3° in methanol (*c*, 1·00) when dried at 100°. Further purification by this method did not alter the rotation significantly. Recrystallization three times from aq. methanol raised it slightly; $[\alpha]_{5461}^{22}$ +187°±3° in methanol (*c*, 1·00).

Some of this material ($[\alpha]_{5461}^{22}$ +187°) was dissolved in aq. methanol and treated with excess of Brady's reagent. The precipitated solid, m.p. 145–151°, had $[\alpha]_{5461}^{23}$ +118±5° in methanol (*c*, 0·20), but attempts to crystallize it led to amorphous products.

SUMMARY

1. On mild acid hydrolysis, tenuazonic acid yields acetic acid and a deacetyl compound.

2. Hydrolysis with stronger acid produces acetic acid, carbon dioxide and a basic ketone, shown to be 3-amino-4-methylhexan-2-one.

3. Ozonolysis of tenuazonic acid, followed by acid hydrolysis, gives acetic acid, oxalic acid and L-isoleucine.

4. It is concluded that tenuazonic acid is α-acetyl-γ-*sec*-butyltetramic acid.

5. The configuration about the two asymmetric centres corresponds to that in L-isoleucine.

6. The crystalline isomer obtained from tenuazonic acid, and previously called *iso*tenuazonic acid, is shown to be a mixture of tenuazonic acid and a diastereoisomer.

7. Tenuazonic acid is believed to be the first substituted tetramic acid isolated from natural sources.

I wish to thank Professor J. H. Birkinshaw for the use of a Hilger and Watts Uvispek spectrophotometer, purchased by means of a grant from the Central Research Fund of the University of London; Miss E. M. Tanner and Mr F. H. Oliver of Parke, Davis and Co. Ltd., Hounslow, Middlesex, for carrying out infrared analyses and microanalyses respectively; and Dr R. N. Lacey, of British Industrial Solvents, Hull, for supplying infrared curves.

REFERENCES

Baker, W., Grice, K. D. & Jansen, A. B. A. (1943). *J. chem. Soc.* p. 241.
Benary, E. (1911). *Ber. disch. chem. Ges.* **44**, 1759.
Birkinshaw, J. H. (1952). *Biochem. J.* **52**, 283.
Birkinshaw, J. H. & Chaplen, P. (1958). *Biochem. J.* **69**, 505.
Birkinshaw, J. H. & Raistrick, H. (1936). *Biochem. J.* **30**, 2194.
Bornwater, J. T. (1912). *Rec. Trav. chim. Pays-Bas*, **31**, 105.
Bracken, A. & Raistrick, H. (1947). *Biochem. J.* **41**, 569.
Clutterbuck, P. W., Haworth, W. N., Raistrick, H., Smith, G. & Stacey, M. (1934). *Biochem. J.* **28**, 94.
Clutterbuck, P. W., Raistrick, H. & Reuter, F. (1935*a*). *Biochem. J.* **29**, 300.
Clutterbuck, P. W., Raistrick, H. & Reuter, F. (1935*b*). *Biochem. J.* **29**, 871.
Clutterbuck, P. W., Raistrick, H. & Reuter, F. (1935*c*). *Biochem. J.* **29**, 1300.
Ehrensvärd, G. (1958). *Chem. Soc. Spec. Publ.* **2**, 14.
Figee, T. (1915). *Rec. Trav. chim. Pays-Bas*, **34**, 289.
Fones, W. S. (1954). *J. Amer. chem. Soc.* **76**, 1377.
Fuson, R. C. & Tullock, C. W. (1934). *J. Amer. chem. Soc.* **56**, 1638.
Greenstein, J. P., Birnbaum, S. M. & Otey, M. C. (1953). *J. biol. Chem.* **204**, 307.
Greenstein, J. P., Levintow, L., Baker, C. G. & White, J. (1951). *J. biol. Chem.* **188**, 647.
Gross, D. & Grodsky, G. (1955). *J. Amer. chem. Soc.* **77**, 1678.
Herbert, R. W. & Hirst, E. L. (1935). *Biochem. J.* **29**, 1881.
Lacey, R. N. (1954). *J. chem. Soc.* p. 850.
Miller, W. T. & Dittman, A. L. (1956). *J. Amer. chem. Soc.* **78**, 2793.
Moore, S. & Stein, W. H. (1951). *J. biol. Chem.* **192**, 663.
Raistrick, H., Stickings, C. E. & Thomas, R. (1953). *Biochem. J.* **55**, 421.
Roedig, A., Becker, H. J., Fugmann, N. & Schoedel, S. (1955). *Liebigs Ann.* **597**, 214.
Rosett, T., Sankhala, R. H., Stickings, C. E., Taylor, M. E. U. & Thomas, R. (1957). *Biochem. J.* **67**, 390.
van Dam-Bakker, A. W. I. (1958). *Nature, Lond.*, **181**, 116.

Oligosaccharide Synthesis in the Banana and its Relationship to the Transferase Activity of Invertase

By R. W. HENDERSON, R. K. MORTON* and W. A. RAWLINSON
Department of Biochemistry, University of Melbourne

(*Received* 15 *August* 1958)

The banana fruit is normally picked when it is firm and green; ripening is then carried out under controlled conditions. It is well known that during such ripening the starch is largely converted into glucose, fructose and sucrose, and water is lost from the skin to the pulp (see Loesecke, 1949). In addition to these sugars, we have detected an oligosaccharide which increases in amount during the ripening process.

The invertase activity of banana pulp has been recognized for many years (Mierau, 1893*a*, *b*). Invertases from a variety of plant tissues have been shown *in vitro* to synthesize oligosaccharides by transglycosidation (see Edelman, 1956; Gottschalk, 1958). It therefore seemed probable that this action was responsible for the formation of banana oligosaccharide *in vivo*.

* Present address: Department of Agricultural Chemistry, University of Adelaide, South Australia.

This paper describes the separation and provisional identification of the above oligosaccharide, along with the partial purification of banana invertase and some studies of its hydrolytic and transferase activities.

MATERIALS AND METHODS

Bananas. Fruits of the variety *Musa cavendishii* (Lamb.), sent by rail from New South Wales, reached Melbourne within a few days of picking in a very firm and quite green condition. They were placed in single layers on wooden trays in a metal bin where ripening was accelerated by exposure for about 16 hr. to an atmosphere of coal gas. The trays were then transferred to a ventilated room which was maintained at 25° and ripening allowed to proceed in darkness. The first sample batch of thirty bananas was taken immediately upon arrival and before gassing; then at intervals of 4–5 days during ripening, similarly sized batches were withdrawn at random and held in a cold room

at 4–10° in separate, sealed containers in an atmosphere of carbon dioxide until analysis. The four stages of ripening selected for study were designated as green, green–yellow, yellow and speckled brown. The total ripening period was about 14 days, after which analysis was immediately commenced. Samples of 30 g. were taken after the bananas had been peeled, minced and the pulp thoroughly mixed. The samples were dehydrated in 97% (v/v) ethanol and then treated as described below.

Soluble sugars and starch. Soluble sugars were extracted by refluxing the dehydrated sample with the original ethanol adjusted to 80% (v/v), for 6 hr., after the method of Barnell (1941). Starch was estimated in the residue as by Pucher & Vickery (1936). Reducing sugars were estimated by the Lane & Eynon (1950) volumetric method. Non-reducing sugars were determined by the same procedure after hydrolysis in 0·1N-HCl at 100° for 15 min.

Sugars. The sample of 6^G-β-fructosylsucrose (for nomenclature see Allen & Bacon, 1956) was kindly supplied by Dr D. Gross, Tate and Lyle Research Laboratory, Keston, Kent. The other sugars were of A.R. quality and their purity, as checked by paper chromatography, was satisfactory.

Yeast invertase. A commercial preparation (British Drug Houses Ltd.) was used.

Banana invertase. A partially purified preparation was obtained as follows. About 1 kg. of pulp from ripe bananas was extracted with 1 l. of 0·05M-sodium pyrophosphate–HCl buffer, pH 7·8, for 2 min. in a Waring Blendor. The dispersion was centrifuged at 2700 *g* for 30 min. The supernatant was removed, brought to pH 7, then solid ammonium sulphate added to 30% saturation and again centrifuged at 2700 *g* for 30 min. The resulting supernatant was increased to 50% (w/v) saturation and centrifuged at 2700 *g* for 30 min. The precipitate was dissolved in about 50 ml. of 0·1M-sodium phosphate buffer, pH 7·2. The brown, somewhat turbid solution was then centrifuged at 1500 *g* for 30 min. and the clear, brown supernatant used as the source of invertase.

Synthesis of oligosaccharides. Yeast invertase was usually incubated for 30 min. with sucrose (10%, w/v) in 0·01M-sodium acetate–acetic acid buffer at pH 5·0 and 25°. Banana invertase was also incubated as described above with sucrose concentrations up to 20% (w/v) for varying times either at room temperature or at 25°. Chromatographic tests of the above solutions, inactivated by boiling, showed no detectable amounts of glucose, fructose or oligosaccharides at zero time of incubation.

Paper chromatography. Whatman no. 3 paper was used throughout. The most generally useful solvent system was butanol–acetic acid–water (5:1:4, by vol.). The other solvent systems used were: propanol–water–aq. NH_3 soln. (16:3:1, by vol.); butanol–ethanol–water (57:13:32, by vol.); butanol–pyridine–water (6:4:3, by vol.).

Spray reagents. The following were used: naphtharesorcinol (Bryson & Mitchell, 1951); aniline hydrogen phthalate (Partridge, 1949); benzidine–trichloroacetic acid (Bacon & Edelman, 1951) and a saturated solution of *p*-anisidine phosphate in moist butanol.

Diazouracil test. The Raybin (1933) test as modified by Feingold, Avigad & Hestrin (1956) was carried out on quantities of sugar about 0·5 mg.

5-Aminouracil was diazotized and stored at 4°; this retained full activity over the 4 weeks' period of test.

Measurement of hydrolysis. The total reducing sugar released by invertase action on sucrose was determined according to King (1951) with the modified Harding & Downs copper reagent, and the cuprous oxide formed was estimated by the blue colour produced with the phosphomolybdic acid solution of Folin & Wu (1920). The inhibitory action of copper on invertase was used to stop the enzymic action at the required time.

EXPERIMENTAL AND RESULTS

In agreement with the findings of earlier workers using other varieties of bananas (see Loesecke, 1949), experiments over several years have shown that there is a substantial decline in the starch content and concurrent increase in both non-reducing and reducing sugars during the ripening process (e.g. see Table 1).

Oligosaccharide in the ripening banana

Fig. 1 shows a chromatogram of the ethanolic extracts of bananas at the four stages of ripening, together with marker spots of glucose, fructose and sucrose. It is seen that these three sugars are present at all stages but increase substantially even at very early stages of ripening when the banana is still quite firm. In addition, there is present a component with R_F less than that of sucrose and whose colour reactions with *p*-anisidine and naphtharesorcinol led to tentative identification as an oligosaccharide consisting predominantly of fructose. This component appears at an early stage and increases during the later stages of ripening.

It seemed likely that the unknown spot was a product formed by invertase action. The reaction products of yeast invertase and sucrose were therefore compared chromatographically with

Table 1. *Reducing and non-reducing sugars in bananas*

Stage of ripeness of bananas	Percentage of fresh pulp			
	Starch	Reducing sugar	Non-reducing sugar	Moisture content
Green	12·1	<0·5	<0·5	71
Yellow–green	8·8	5·0	5·1	74
Yellow	1·9	8·4	8·9	74
Speckled brown	0·9	10·3	8·2	75

ethanolic extracts of banana and it was observed that there was an oligosaccharide formed by the yeast invertase in a position identical with that of the unknown component obtained from the banana.

Separation and crystallization of the oligosaccharide

The ethanolic extract of banana was applied to the paper as a streak and then run in the usual butanol–acetic acid solvent. The positions of the sugars were defined by spraying narrow strips cut down the long edges of the paper. The paper was then cut across just behind the sucrose streak to obtain a strip containing the unknown saccharide which was eluted with water into about 0·3 ml. of solution and concentrated *in vacuo* at room temperature; on being taken slowly to dryness a crystalline residue was obtained. This residue was rechecked by paper chromatography and one spot only was obtained (see Fig. 2).

Identification of the oligosaccharide

The banana oligosaccharide was hydrolysed in a sealed tube for 1 hr. at 100° in 0·6N-HCl. Tested chromatographically, the hydrolysate showed only fructose and glucose. The ratio of these two sugars was then determined on paper, chromatographically, by the micromethod of Gottschalk & Ada (1956). For estimation of fructose against fructose standards the naphtharesorcinol-spray reagent was used, and, for glucose, aniline hydrogen phthalate was used. The ratio of fructose to glucose was found to be 2:1 (±10%).

The Raybin (1933) diazouracil test on the compound gave a deep yellow–green (positive reaction). Addition of magnesium sulphate to this coloured solution produced a stable blue precipitate.

Chromatographic comparison of the oligosaccharide with authentic 6^G-β-fructosylsucrose in the four solvent systems listed revealed identical R_F values and colour shades for the two substances.

Transferase activity of banana invertase in vitro

Paper chromatography of the products of the banana invertase–sucrose reaction showed the presence of three spots with R_F values less than that of sucrose.

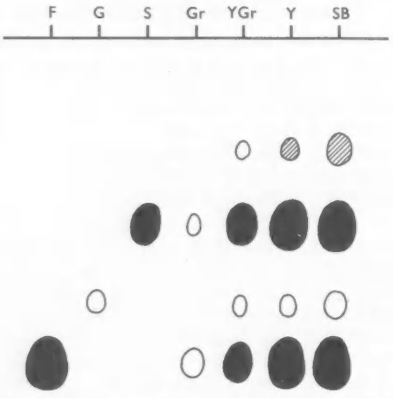


Fig. 1. Chromatogram of equal volumes (about 5 μl.) of extracts of 30 g. of banana pulp (fresh weight) in 80% (v/v) ethanol (final volume 250 ml.), at the stages of ripeness corresponding to green (Gr), yellow–green (YGr), yellow (Y) and speckled brown (SB). Reference sugars consisted of equal volumes (about 5 μl.) of 2·5% (w/v) fructose (F), 2·5% (w/v) sucrose (S) and 5% (w/v) glucose (G). The chromatograms were run for 18 hr. in butanol–acetic acid–water (5:1:4, by vol.) at 25°, dried and sprayed with 0·2% naphtharesorcinol solution (Bryson & Mitchell, 1951). The degree of shading indicates approximately the intensity of coloration of the spots.

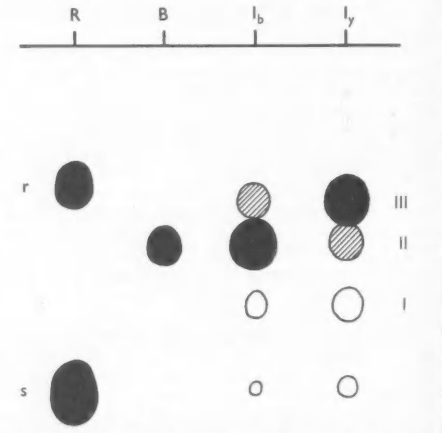


Fig. 2. Chromatogram of the oligosaccharides obtained from ripe bananas (B), the banana invertase–sucrose reaction (I_b) *in vitro* and the yeast invertase–sucrose reaction (I_y) *in vitro*. These products were separated chromatographically in butanol–acetic acid–water from glucose, fructose and sucrose (see Fig. 1), eluted, concentrated and run again in the same solvent system. B, Crystalline oligosaccharide component from ethanolic extract of banana. I_b, Partially purified banana invertase (see text) incubated with 20% (w/v) sucrose in 0·02M-sodium phosphate–0·01M-sodium citrate buffer, pH 4·0, for 120 min. at 25°; action was stopped by boiling. I_y, Yeast invertase incubated with 10% (w/v) sucrose in 0·01M-sodium acetate–acetic acid buffer, pH 5·0, for 30 min. at 25°; action was stopped by boiling; spots I, II and III are labelled after Bacon & Edelman (1950). R, Reference mixture of 2·5% (w/v) raffinose (r) and 2·5% (w/v) sucrose (s). The degree of shading indicates approximately the intensity of coloration of the spots.

The oligosaccharides produced by this reaction and also those produced by the yeast invertase–sucrose reaction were separated from the mono- and di-saccharides by paper chromatography, eluted and concentrated as already described for the oligosaccharide from the ethanolic extract of banana. Chromatographic comparison of the oligosaccharides from these sources (see Fig. 2) showed that the three spots produced by the banana invertase–sucrose reaction *in vitro* coincided with the spots numbered I, II and III by Bacon & Edelman (1950) from the yeast invertase–sucrose reaction. The single oligosaccharide spot from the banana extract had an R_F value identical with spot II (banana), which was the main component synthesized by the banana invertase *in vitro*. The banana invertase–sucrose reaction always gave a predominant spot II under the varying conditions of pH, buffer, temperature and time used, as compared with a predominant spot III in yeast invertase–sucrose tested similarly.

K_m values for banana and yeast invertases acting on sucrose

Preliminary experiments have shown that the pH optimum of banana invertase is at about pH 4. As with yeast invertase, the pH–activity curve has a rather broad peak but falls away steeply on each side. K_m values at various pH values so far determined reveal a large variation between banana and yeast invertases, e.g. at pH 5·1 the values were 0·5 and 20·0 mM respectively.

DISCUSSION

The results shown in Table 1 are in agreement with the findings of earlier workers (see Loesecke, 1949) that starch is converted into glucose, fructose and sucrose during ripening of the banana. In addition to these mono- and di-saccharides it is clear from the preceding section that at least one oligosaccharide is also formed during ripening. All these sugars appear at a very early stage when the fruit is quite firm and the cells intact, and they increase throughout ripening.

The oligosaccharide found in the banana appears to be identical with one formed by the action of banana invertase on sucrose *in vitro* and also with a component oligosaccharide of spot II from the yeast invertase–sucrose reaction (see Fig. 2). Spot II from the latter reaction has been separated chromatographically by Bacon (1954) using a charcoal–Celite column, and shown to consist of two trisaccharides, each of two fructose and one glucose residue. These two oligosaccharides, designated by Bacon as II_1 and II_2, have been investigated by several workers (see Edelman, 1956; Gottschalk, 1958) and the structures shown to be: II_1, 1-β-fructosylsucrose; II_2, 6^G-β-fructosylsucrose. The evidence obtained shows that the ripening banana contains 6^G-β-fructosylsucrose, which increases in amount along with sucrose, glucose and fructose during ripening. Banana invertase was shown to catalyse the formation of oligosaccharides *in vitro* and it is concluded that the banana oligosaccharide also is formed *in vivo* by transfructosylation from sucrose to other sucrose molecules (acting as acceptors) and catalysed by the same enzyme.

Although there have been numerous examples of such transfructosylation (and several of transglucosylation) by invertases since the discovery of the transferring action of these hydrolases *in vitro* (see Gottschalk, 1958), to our knowledge there has been no previous demonstration that this action can occur *in vivo* in higher plants. It might be argued that this reaction occurs only in cells which have undergone some type of degeneration. However, the anatomical evidence of early investigators (see Loesecke, 1949) strongly suggests that most cells of the ripening banana remain quite intact, with little or no degeneration of the cytoplasm, until the very last stages of ripening, and it is quite clear that the oligosaccharide component makes its appearance long before the banana reaches a 'mushy' stage.

The banana invertase–sucrose reaction produces, *in vitro*, in addition to the spot II (banana) already discussed, spots which are coincident with spots I and III from the yeast invertase–sucrose reaction. These latter have been shown (see Gottschalk, 1958) to be fructosylglucose and 6^F-β-fructosylsucrose respectively and it is likely that the sugars produced by the banana invertase are the same. It is not clear why only trisaccharide fraction II is found in the ethanolic extract of the banana whereas a number of different oligosaccharides are formed by banana invertase action on sucrose *in vitro*. It is possible that the other products found *in vitro* are also formed *in vivo* but are preferentially metabolized.

SUMMARY

1. The starch, sucrose and monosaccharide content of bananas of the variety *Musa cavendishii* has been investigated during ripening. In agreement with earlier workers using similar varieties it has been observed that starch is converted into glucose, fructose and sucrose during this process.

2. A trisaccharide also formed during the ripening process has been crystallized from banana extracts and provisionally identified as 6^G-β-fructosylsucrose.

3. The hydrolytic and transferase activities of banana invertase have been demonstrated *in vitro*.

The oligosaccharides formed have been compared chromatographically with the above trisaccharide and those components of the yeast invertase–sucrose reaction having similar R_F values. Conclusions are drawn about the transferase activity of banana invertase *in vivo*.

We are indebted to Dr A. Gottschalk of the Walter and Eliza Hall Institute of Research for his helpful discussions.

REFERENCES

Allen, P. J. & Bacon, J. S. D. (1956). *Biochem. J.* **63**, 200.
Bacon, J. S. D. (1954). *Biochem. J.* **57**, 320.
Bacon, J. S. D. & Edelman, J. (1950). *Arch. Biochem.* **28**, 467.
Bacon, J. S. D. & Edelman, J. (1951). *Biochem. J.* **48**, 114.
Barnell, H. R. (1941). *Ann. Bot., N.S.*, **5**, 217.
Bryson, J. L. & Mitchell, T. J. (1951). *Nature, Lond.*, **167**, 864.
Edelman, J. (1956). *Advanc. Enzymol.* **17**, 189.
Feingold, D. S., Avigad, G. & Hestrin, S. (1956). *Biochem. J.* **64**, 351.
Folin, O. & Wu, H. (1920). *J. biol. Chem.* **41**, 367.
Gottschalk, A. (1958). In *Handbuch der Pflanzenphysiologie*, vol. 2, p. 87. Ed. by Ruhland, W. Berlin: Springer-Verlag.
Gottschalk, A. & Ada, G. L. (1956). *Biochem. J.* **62**, 681.
King, E. J. (1951). In *Microanalysis in Medical Biochemistry*, 2nd ed., p. 23. London: J. and A. Churchill.
Lane, J. H. & Eynon, L. (1950). In *Official and Tentative Methods of Analysis*, 7th ed., p. 506. Association of Official Agricultural Chemistry.
Loesecke, H. W. von (1949). In *Bananas-Chemistry, Physiology, Technology*. New York: Interscience Publishers Inc.
Mierau, F. (1893*a*). *Chemikerztg.* **17**, 1002.
Mierau, F. (1893*b*). *Chemikerztg.* **17**, 1283.
Partridge, S. M. (1949). *Nature, Lond.*, **164**, 443.
Pucher, G. W. & Vickery, H. B. (1936). *Industr. Engng Chem., (Anal.)*, **8**, 92.
Raybin, H. W. (1933). *J. Amer. chem. Soc.* **55**, 2603.

Fungal detoxication

4. METABOLISM OF 2-METHOXYNAPHTHALENE BY *ASPERGILLUS NIGER**

By R. J. W. BYRDE, D. F. DOWNING and D. WOODCOCK
Long Ashton Research Station, Bristol University

(*Received* 2 *July* 1958)

The metabolism of naphthalene by a variety of animals has been extensively studied by Young and by Boyland and their respective co-workers. The initial product in rats was shown by Young (1947) to be D-*trans*-1:2-dihydro-1:2-dihydroxynaphthalene, both the diol and 1-naphthol and their conjugates having been isolated from urine. There appears to be little breakdown of the naphthalene ring, however (Boyland & Wiltshire, 1953), which is in sharp contrast with the degradation of naphthalene compounds by soil micro-organisms (Walker & Wiltshire, 1953, 1955; Treccani, Walker & Wiltshire, 1954; Canonica, Fiecchi & Treccani, 1957).

In previous work by Byrde, Harris & Woodcock (1956) the metabolism of ω-(2-naphthyloxy)-*n*-alkylcarboxylic acids by *Aspergillus niger* van Tiegh. was studied, the main features being nuclear hydroxylation, together with β-oxidation of all side chains except those of 2-naphthyloxyacetic acid and β-(2-naphthyloxy)propionic acid. In a later paper (Byrde & Woodcock, 1958) a similar investigation, in which *Sclerotinia laxa* Aderh. & Ruhl. was used with the same series of acids, showed β-oxidation to be the main process involved, nuclear hydroxylation being virtually absent. There was little evidence of ring breakdown of the naphthyloxy acid with either fungus, and the present investigations with 2-methoxynaphthalene as a non-polar sole-carbon source were made in order to examine the effect of this change on ring-fission.

* Part 3 Byrde & Woodcock (1958).

EXPERIMENTAL

All m.p.'s are uncorrected. Ultraviolet-absorption spectra were determined in methanol solution with a Unicam SP. 500 spectrophotometer. Infrared-absorption spectra were determined on mulls prepared by intimately grinding the samples with Nujol, by using a Hilger 800 spectrophotometer.

MATERIALS

2-Methoxynaphthalene (nerolin). This was used as obtained from British Drug Houses Ltd., m.p. 73–74°.

2-Ethoxynaphthalene. This was prepared from 2-naphthol with diethyl sulphate and NaOH. Crystallized from ethanol, it had m.p. 35–36°.

4-Methoxysalicylic acid. This was prepared from 2:4-dihydroxybenzoic acid by the method of Gomberg & Johnson (1917). It crystallized from water in long silky needles, m.p. 157–158° (Found: C, 57·1; H, 4·8. Calc. for $C_8H_8O_4$: C, 57·1; H, 4·7%). Gomberg & Johnson (1917) give m.p. 157°. The ultraviolet-absorption spectrum showed

the following maxima: 214 mμ ($\epsilon_{max.}$ 18 780), 254 mμ ($\epsilon_{max.}$ 12 260) and 295 mμ ($\epsilon_{max.}$ 5590).

The infrared spectrum showed the following main absorption maxima (cm.$^{-1}$): 2909, 1645, 1465, 1270.

4-Ethoxysalicylic acid. Prepared similarly to the corresponding methoxy compound, by using diethyl sulphate, this acid crystallized from water in silky white needles, m.p. 152–153° (Found: C, 59·1; H, 5·4. Calc. for $C_9H_{10}O_4$: C, 59·3; H, 5·5%). Perkin (1895) gives m.p. 153–154°. The infrared-absorption spectrum showed the following absorption maxima (cm.$^{-1}$): 2932, 1655, 1465, 1275.

4-Methoxyphthalic acid. 4-Hydroxyphthalic acid, prepared by the method of Baeyer (1877), was methylated with dimethyl sulphate and NaOH. The methoxy acid crystallized from methanol in prisms, m.p. 165–166°. Bentley & Weizmann (1907) give m.p. 170°.

5-Amino-2-methoxynaphthalene. A solution of 2-methoxy-5-nitronaphthalene (1·8 g.), prepared as described by James & Woodcock (1951), in tetrahydrofuran (10 ml.) was shaken in hydrogen with Raney nickel; it rapidly absorbed the theoretical amount of gas. The blue fluorescent solution was filtered and evaporated under reduced pressure. The residual red oil was dissolved in ether and shaken with 10% (v/v) HCl; the acid solution was made alkaline with NaOH and the product was isolated with ether. The ethereal solution was dried (Na_2SO_4) and the solvent removed, leaving a red oil (1·1 g., yield 72%) which crystallized from aqueous methanol (charcoal) or light petroleum (b.p. 60–80°) in rosettes of prisms, m.p. 57–58° (Found: C, 76·5; H, 6·2; N, 8·2. Calc. for $C_{11}H_{11}ON$: C, 76·3; H, 6·3; N, 8·1%). On one occasion a dimorphic form, m.p. 73–74°, was obtained, and a solution of the lower-m.p. form gave the higher-m.p. form by seeding. Cohen, Cook, Hewett & Girard (1934) report needles, m.p. 73–74°; Campbell, La Forge & Campbell (1949) give m.p. 64–66°. *5-Acetamido-2-methoxynaphthalene* crystallized from water in clusters of needles, m.p. 124–125°, or from aqueous methanol in plates, m.p. 139–140° [Found (sample m.p. 124–125°): C, 72·8; H, 6·3; N, 6·6. Calc. for $C_{13}H_{13}O_2N$: C, 72·6; H, 6·1; N, 6·5%]. Cohen *et al.* (1934) give m.p. 142·5–143·5°.

5-Hydroxy-2-methoxynaphthalene. A solution of 5-amino-2-methoxynaphthalene (0·5 g.) in acetic acid (2·6 ml.) was stirred with H_2SO_4 (1·3 ml.) and water (1·3 ml.), and after cooling to 10° a solution of $NaNO_2$ (0·26 g.) in water (1 ml.) was added slowly dropwise. After stirring for 2 hr. the mixture was poured into boiling 5% (v/v) H_2SO_4 (80 ml.) and filtered hot, the filtrate cooled, extracted with ether and the ethereal layer shaken with 10% (w/v) NaOH. The alkaline washings were acidified with HCl and extracted with ether, and the ethereal extract dried (Na_2SO_4). Removal of the solvent gave a brown oil which crystallized from light petroleum (b.p. 40–60°) in colourless plates (0·35 g., 70%) m.p. 85° (Found: C, 76·0; H, 5·6. $C_{11}H_{10}O_2$ requires C, 75·9; H, 5·7%). This compound was reported as an oil, b.p. 185°/12 mm. Hg (Baeyer & Co., 1921).

6-Hydroxy-2-methoxynaphthalene. This was prepared as described by Downing & Woodcock (1958).

7-Hydroxy-2-methoxynaphthalene. This was prepared from 2:7-dihydroxynaphthalene according to the method of Fischer & Hammerschmidt (1916).

8-Nitro-2-methoxynaphthalene. 8-Nitro-2-naphthol (1·8 g.) (Friedlander & Szymanski, 1892) was methylated with dimethyl sulphate and NaOH. The oil obtained crystallized from aqueous methanol in bright-yellow needles (1·5 g., 77%), m.p. 74–75° (Found: C, 65·2; H, 4·4; N, 7·1. Calc. for $C_{11}H_9O_3N$: C, 65·0; H, 4·4; N, 6·9%). Friedlander & Szymanski (1892) give m.p. 69°.

8-Amino-2-methoxynaphthalene. 2-Methoxy-8-nitronaphthalene (1 g.) was reduced as described for the 5-amino compound. The resultant red oil crystallized from aqueous methanol (charcoal) in prismatic plates (0·8 g., 89%), m.p. 79–80°. Bachmann & Horton (1947) give m.p. 79–80°.

8-Hydroxy-2-methoxynaphthalene. This was prepared from 8-amino-2-methoxynaphthalene as described for the 5-hydroxy compound. The red, oily product crystallized from light petroleum (b.p. 60–80°) as white plates (0·25 g., 50%), m.p. 100–102° (Found: C, 75·5; H, 5·6. Calc. for $C_{11}H_{10}O_2$: C, 75·9; H, 5·7%). Angeletti (1929) gives m.p. approx. 122–124°, after sintering at about 60°.

2-Carboxy-5-methoxycinnamic acid. This was prepared as described by Downing & Woodcock (1958).

Methods

Replacement-culture technique and chromatographic examination of metabolites. The general method followed that previously described (Byrde *et al.* 1956), except that the substrate was incubated with the mycelial mats of *A. niger* for periods of 1–7 days before being continuously extracted with ether. In view of the volatility of 2-methoxy- and 2-ethoxy-naphthalene, the amounts lost during incubation and extraction were estimated by including a flask containing substrate, but no fungus.

The chromatographic solvents used for examining these extracts were butan-1-ol–ethanol–3N-NH_3 (4:1:5, by vol.) (solvent A), aqueous 0·1N-NH_3 (solvent B) and benzene–propionic acid–water (10:7:1) (solvent C). Solvent B was useful for separation of naphthols which ran too near the solvent front in solvent A. After drying and exposure under ultraviolet light (wavelength of maximum emission 2539 Å), fluorescent spots were marked and the paper was then sprayed with either 2% aqueous $FeCl_3$, a 0·02% solution of *p*-nitrobenzenediazonium fluoroborate (Freeman, 1952) in acetone, or B.D.H. universal indicator, which had been previously adjusted to pH 9–10 with NaOH.

Fungitoxicity in vitro. Comparisons of fungitoxicity were carried out by the method described by Byrde & Woodcock (1957), the medium being adjusted to approximately pH 2·0 after sterilization, by aseptic addition of H_3PO_4 (10%, v/v).

RESULTS

Metabolism of 2-methoxynaphthalene

Isolation of 4-methoxysalicylic acid. In a typical experiment in which 2-methoxynaphthalene (197·5 mg.) was incubated with *A. niger*, a total of 65 mg. was lost by volatilization in the incubator or recovered unchanged and 78 mg. of material soluble in aq. $NaHCO_3$ was obtained. Chromatographic examination of the acidic extract showed the presence of a strong acidic spot, R_g 0·59 (solvent A), R_F 0·69 (solvent B), R_F 0·78 (solvent C), which gave a violet colour with $FeCl_3$ and an orange–brown colour with *p*-nitrobenzenediazonium fluoroborate. The residue obtained by removal of the solvent from the ethereal extract

crystallized from water in fawn, felted needles, m.p. 156–157° (Found: C, 57·35; H, 4·7%). The ultraviolet-absorption spectrum showed maxima at 214 mμ ($\epsilon_{max.}$ 20 030), 252 mμ ($\epsilon_{max.}$ 12 260) and 295 mμ ($\epsilon_{max.}$ 5410); the infrared spectrum showed main maxima (cm.$^{-1}$) as follows: 2932, 1655, 1465, 1270 mμ. This acid did not depress the m.p. of authentic 4-methoxysalicylic acid, and the ultraviolet- and infrared-absorption spectra are virtually identical. By chromatography only two weak spots were detectable in addition to that formed by 4-methoxysalicylic acid. The first, R_F 0·59 (i.e. coincident with 4-methoxysalicylic acid in solvent A) and R_F 0·74 (solvent C), gave an intense blue fluorescence under ultraviolet light (wavelength of maximum emission 2539 Å), whereas the second, R_F 0·84 (solvent A), which had a mauve fluorescence, appeared very similar to 7-methoxycoumarin, R_F 0·88 (solvent A). Attempts are being made to confirm its identity.

Attempted isolation of intermediates. The method of Walker & Wiltshire (1955) for the isolation of chloronaphthalenediol was modified as follows: The reaction mixture obtained after incubation for 24 hr. was stirred for 2 hr. with activated charcoal (1 g./l.); the charcoal was filtered off, dried in air and then extracted with a mixture of ethanol and benzene (1:1, v/v) in a Soxhlet apparatus for 8 hr. The solvents were removed under reduced pressure and the residue was treated with water and filtered and the aqueous solution was extracted repeatedly with ether. The combined extracts were washed with $NaHCO_3$, then with water, and were dried (Na_2SO_4) and the solvent was removed. The residual brown oil (*X*) did not crystallize from any of a variety of solvents. Chromatographic analysis indicated that it contained a phenolic compound (*Y*), R_F 0·48 (solvent B), which was different from any of the naphthols listed in Table 1.

Acidification and ether extraction of the $NaHCO_3$ washings gave 4-methoxysalicylic acid (identified chromatographically).

Metabolism of potential precursors of 4-methoxysalicylic acid. No 4-methoxysalicylic acid was detected when the following substrates (mM) were incubated with *A. niger* for 7-day periods: 6-hydroxy-2-methoxynaphthalene, 4-methoxybenzoic acid, 4-methoxyphthalic acid and 2-carboxy-5-methoxycinnamic acid. The non-acidic fraction (*X*) obtained after incubation of 2-methoxynaphthalene with *A. niger* for 24 hr. also failed to yield 4-methoxysalicylic acid when incubated for 3 days with a fresh mycelial mat of *A. niger*. In another experiment, salicylic acid was not detected when L-*trans*-1:2-dihydro-1:2-dihydroxynaphthalene (kindly supplied by Dr N. Walker, Rothamsted Experimental Station) was incubated with *A. niger* for 2 days.

Metabolism of 2-ethoxynaphthalene

In a typical experiment in which 2-ethoxynaphthalene (215 mg.) was incubated with *A. niger*, a total of 46 mg. was lost by volatilization or recovered unchanged and 49 mg. of aq. $NaHCO_3$-soluble material was obtained. Chromatographic examination of the ethereal extract showed the presence of an acid, R_F 0·68 (solvent A), with the properties expected of 4-ethoxysalicylic acid. The residue obtained by removal of the solvent crystallized from aqueous methanol (charcoal) in needles, m.p. 152–153°, undepressed by admixture with authentic 4-ethoxysalicylic acid, m.p. 152–153°. The infrared-absorption spectrum, which showed maxima (cm.$^{-1}$) at 2932, 1655, 1465 and 1275, was identical with that of 4-ethoxysalicylic acid.

Fungitoxicity in vitro

Table 2 shows the mean growth rate (mm./day) at pH 2·0 of cultures of *A. niger* in the presence of 2-methoxynaphthalene, 2-ethoxynaphthalene and the corresponding salicylic acid detoxication products. Probit analysis of the quantitative data (Finney, 1952) is summarized in Table 3.

DISCUSSION

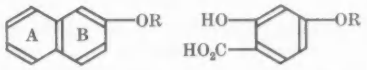
Although there was little evidence of ring-fission in the metabolism of ω-(2-naphthyloxy)-*n*-alkylcarboxylic acids by *Aspergillus niger* and *Sclerotinia laxa* (Byrde *et al.* 1956; Byrde & Woodcock, 1958),

Table 1. *Chromatographic data of naphthols*

Compounds were run in aqueous 0·1 N-NH_3 (solvent B) by downward solvent flow. After drying and exposure under ultraviolet light (wavelength of maximum emission, 2539 Å), fluorescent spots were marked and the paper was then sprayed with a 0·02% solution of *p*-nitrobenzenediazonium fluoroborate (Freeman, 1952) in acetone.

Compound	R_F	Fluorescence in u.v. light	Colour with diazo spray
Y	0·48	Blue	Deep pink
2-Naphthol	0·48	Purple	Salmon pink
5-Hydroxy-2-methoxynaphthalene	0·39	Deep blue	Light orange–brown
6-Hydroxy-2-methoxynaphthalene	0·28	Purple	Mauve–pink
7-Hydroxy-2-methoxynaphthalene	0·36	Purple	Light orange–brown
8-Hydroxy-2-methoxynaphthalene	0·43	Pale blue	Deep mauve–pink

this process is essential for the formation of 4-methoxysalicylic acid (II; R = CH_3), which is the only metabolic product of 2-methoxynaphthalene (I; R = CH_3) with *A. niger* which has been isolated.



(I) (II)

Considering oxidative fission of 2-methoxynaphthalene, it would have seemed likely that the more active ring B should undergo initial attack, but the isolation of 4-methoxysalicylic acid, together with the formation of 4-ethoxysalicylic acid (II; R = C_2H_5) from 2-ethoxynaphthalene (I; R = C_2H_5), implies that ring A has undergone degradation and was therefore more highly activated than ring B. The presence of the hydroxyl group in the final products suggests that this substitution must occur after ring-fission, since otherwise ring B would have been more active originally and hence degraded with the loss of the alkoxyl group. Hydroxylation after ring-fission is a feature of the scheme put forward by Fernley & Evans (1958) for the end-ring attack on naphthalene by soil Pseudomonads, the metabolic pathway to salicylic acid being by way of D-*trans*-1:2-dihydro-1:2-dihydroxynaphthalene, 1:2-dihydroxynaphthalene, *o*-carboxy-*cis*-cinnamic acid and *o*-hydroxycinnamic acid. A similar route via 2-hydroxy-4-methoxy-*cis*-cinnamic acid would seem to be the most likely for the formation of 4-methoxysalicylic acid from 2-methoxynaphthalene, and would explain the formation of 7-methoxycoumarin, the presence of which has been indicated chromatographically. An alternative pathway by way of 5:6-dihydro-5:6-dihydroxy-2-methoxynaphthalene, 5:6-dihydroxy-2-methoxynaphthalene and 2-carboxy-5-methoxycinnamic acid, which thus involves the ultimate replacement of $-CH:CH\cdot CO_2H$ by $-OH$, seems less likely, although the failure of 2-carboxy-5-methoxycinnamic acid to give 4-methoxysalicylic acid when incubated with *A. niger* could also be explained by stereospecificity of the fungal enzyme (cf. Evans & Smith, 1951). The possibility that 4-methoxyphthalic acid is involved in either route seems unlikely, since we were unable to detect the formation of 4-methoxysalicylic acid from 4-methoxyphthalic acid (cf. Henderson, 1957).

The successful isolation of a diol as a primary metabolic product has been reported from naphthalene (Walker & Wiltshire, 1953), 1-chloronaphthalene (Walker & Wiltshire, 1955) and 2-chloro- and 2-methyl-naphthalene (Canonica *et al.* 1957). The failure of Canonica *et al.* (1957) to isolate a diol intermediate from 1-methylnaphthalene by using *Pseudomonas desmolytica* has been followed by our

Table 2. *Mean growth rate of* A. niger *mycelium in the presence of 2-methoxynaphthalene, 2-ethoxynaphthalene and their detoxication products*

Conditions were as described in the text.

Test no.	Compound added	Concn. (μM)	Mean rate of growth (mm./day)
1	2-Methoxynaphthalene	50	8·69
		100	8·00
		200	5·25
		400	2·56
		800	1·31
		1600	1·37
	4-Methoxysalicylic acid	70	13·00
		100	11·56
		140	8·37
		200	2·31
		280	0·00
		400	0·37
	Control	—	13·94
2	2-Ethoxynaphthalene	50	10·50
		100	10·00
		200	8·81
		400	6·00
		800	0·56
		1600	0·13
	4-Ethoxysalicylic acid	35	9·94
		50	8·31
		70	7·06
		100	4·75
		140	0·00
		200	0·00
	Control	—	11·44

Table 3. *Dose–response data derived from Table 2*

Test no.	Compound	Equation of probit regression line	10^5 ED_{50} (M)	Slope of probit regression line
1	2-Methoxynaphthalene	$Y = 6{\cdot}39 - 1{\cdot}32x$	11·32 (2·58–21·52)	$-1{\cdot}32 \pm 0{\cdot}42$
	4-Methoxysalicylic acid	$Y = 12{\cdot}17 - 6{\cdot}12x$	14·86 (10·32–18·52)	$-6{\cdot}12 \pm 1{\cdot}71$
2	2-Ethoxynaphthalene	$Y = 9{\cdot}98 - 3{\cdot}16x$	37·58 (27·60–50·46)	$-3{\cdot}16 \pm 0{\cdot}55$
	4-Ethoxysalicylic acid	$Y = 9{\cdot}26 - 4{\cdot}64x$	8·25 (6·60–9·73)	$-4{\cdot}64 \pm 0{\cdot}66$

inability to isolate the expected diol from 2-methoxynaphthalene either as such or after conversion into hydroxymethoxynaphthalenes by acid treatment (cf. Walker & Wiltshire, 1955). Unlike Walker & Wiltshire (1953), Murphy & Stone (1955) found no evidence of the presence of D-*trans*-1:2-dihydro-1:2-dihydroxynaphthalene from a culture of a strain of *Pseudomonas* growing on naphthalene and concluded that possibly the 'diol' was utilized as rapidly as it was formed and consequently not detected in the medium. This is possibly what happens if a diol is formed from 2-methoxynaphthalene.

The toxicity studies were carried out at pH 2·0, where the salicylic acids would be expected to be present as undissociated molecules. The breakdown of 2-methoxynaphthalene to 4-methoxysalicylic acid was accompanied by some decrease in fungistatic activity to *A. niger* at the 50% response level. On the other hand, 4-ethoxysalicylic acid was more active than ethoxynaphthalene, and provides an example of a breakdown product (formed in substantial amount) which is more active than the initial material (cf. Williams, 1947). It seems likely that the low aqueous solubility of the two naphthalenes is a limiting factor in their fungistatic activity. In the corresponding salicylic acids, however, adequate solubility in water is assured by the presence of the polar groups, and an increase in the size of the lipophilic alkyloxy grouping would be expected to increase distribution to the biophase, and hence to increase fungistatic activity (Albert, 1951). It is also possible that chelation may play some part in the action of these acids at the higher pH values within the cell.

SUMMARY

1. By the use of a replacement-culture technique with *Aspergillus niger* 4-methoxysalicylic acid was isolated as a metabolic product from 2-methoxynaphthalene, and 4-ethoxysalicylic acid similarly from 2-ethoxynaphthalene.

2. 4-Methoxybenzoic acid, 4-methoxyphthalic acid, 2-carboxy-5-methoxycinnamic acid and 6-hydroxy-2-methoxynaphthalene did not produce 4-methoxysalicylic acid when incubated with *A. niger*.

3. Although the fungistatic activity (based on ED_{50} values at pH 2·0) of 4-methoxysalicylic acid was less than that of 2-methoxynaphthalene, 4-ethoxysalicylic acid was more active than the corresponding 2-ethoxynaphthalene.

The authors wish to thank Miss M. Waller and Miss N. Waugh for valuable technical assistance, Mr D. R. Clifford for the microanalyses and the ultraviolet spectra, and Dr E. R. Sayer for the infrared spectra.

REFERENCES

Albert, A. (1951). *Selective Toxicity with Special Reference to Chemotherapy*. London: Methuen and Co. Ltd.
Angeletti, A. (1929). *Gazz. chim. ital.* **59**, 851.
Bachmann, W. E. & Horton, W. J. (1947). *J. Amer. chem. Soc.* **69**, 58.
Baeyer, A. (1877). *Ber. dtsch. chem. Ges.* **10**, 1079.
Baeyer and Co. (1921). DRP 298 098. Cited in *Chem. Zbl.* (1921), II, 504.
Bentley, W. H. & Weizmann, Ch. (1907). *J. chem. Soc.* **91**, 98.
Boyland, E. & Wiltshire, G. H. (1953). *Biochem. J.* **53**, 636.
Byrde, R. J. W., Harris, J. F. & Woodcock, D. (1956). *Biochem. J.* **64**, 154.
Byrde, R. J. W. & Woodcock, D. (1957). *Biochem. J.* **65**, 682.
Byrde, R. J. W. & Woodcock, D. (1958). *Biochem. J.* **69**, 19.
Campbell, K. N., La Forge, R. A. & Campbell, B. K. (1949). *J. org. Chem.* **14**, 346.
Canonica, L., Fiecchi, A. & Treccani, V. (1957). *R.C. Ist. lombardo*, **91**, I, 119. Cited in *Chem. Abstr.* (1958). **52**, 12 988.
Cohen, A., Cook, J. W., Hewett, C. L. & Girard, A. (1934). *J. chem. Soc.* p. 653.
Downing, D. F. & Woodcock, D. (1958). *J. chem. Soc.* p. 531.
Evans, W. C. & Smith, B. S. W. (1951). *Biochem. J.* **49**, x.
Fernley, H. N. & Evans, W. C. (1958). *Nature, Lond.*, **182**, 373.
Finney, D. J. (1952). *Probit Analysis*, p. 183. Cambridge University Press.
Fischer, O. & Hammerschmidt, C. B. (1916). *J. prakt. Chem.* **94**, 24.
Freeman, J. H. (1952). *Analyt. Chem.* **24**, 955.
Friedlander, P. & Szymanski, St. (1892). *Ber. dtsch. chem. Ges.* **25**, 2079.
Gomberg, M. & Johnson, L. C. (1917). *J. Amer. chem. Soc.* **39**, 1687.
Henderson, M. E. K. (1957). *J. gen. Microbiol.* **16**, 686.
James, P. M. & Woodcock, D. (1951). *J. chem. Soc.* p. 3418.
Murphy, J. F. & Stone, R. W. (1955). *Canad. J. Microbiol.* **1**, 579.
Perkin, A. G. (1895). *J. chem. Soc.* **67**, 995.
Treccani, V., Walker, N. & Wiltshire, G. H. (1954). *J. gen. Microbiol.* **11**, 341.
Walker, N. & Wiltshire, G. H. (1953). *J. gen. Microbiol.* **8**, 273.
Walker, N. & Wiltshire, G. H. (1955). *J. gen. Microbiol.* **12**, 478.
Williams, R. T. (1947). *Detoxication Mechanisms*, p. 241. London: Chapman and Hall Ltd.
Young, L. (1947). *Biochem. J.* **41**, 417.

The Mechanism of Ficin-Catalysed Reactions

By B. R. HAMMOND and H. GUTFREUND
National Institute for Research in Dairying, Shinfield, near Reading, Berks

(*Received* 27 *October* 1958)

During the last few years a number of hydrolytic and acyl-transfer enzymes have been studied in detail and marked similarities in their reaction mechanisms have been found. Cholinesterase (Wilson & Calib, 1956), chymotrypsin (Gutfreund & Sturtevant, 1956) and trypsin (Gutfreund, 1955*a*) have been shown to react with their substrates by a three-step mechanism involving an initial rapid adsorption of the substrate and a subsequent chemical reaction with an active site consisting of a histidine imidazole and a serine hydroxyl group. More recent studies on phosphoglucomutase (Koshland & Erwin, 1957) and on thrombin (Gladner & Laki, 1958) revealed that these enzymes also have serine hydroxyl groups as a component of their active sites. Gutfreund (1955*b*) and Bernhard & Gutfreund (1956) proposed a catalytic mechanism for ficin which is almost identical with that proposed for papain-catalysed reactions by Kimmel & Smith (1957). This mechanism again involves an initial adsorption process and a subsequent chemical reaction with the catalytic groups. The catalytic site of ficin contains a sulphydryl group which is acylated during the reaction between enzyme and substrate. This paper describes experiments which were carried out to extend the studies of the properties and reactivity of the active site of ficin, which were begun by Bernhard & Gutfreund (1956).

EXPERIMENTAL

Methods

The constant-pH titration procedure as adapted by Hammond & Gutfreund (1955) was used to follow the rate of enzyme-catalysed ester hydrolysis reactions. Standard conditions for the assay of ficin activity are: temperature, 25±0·05°; 15 ml. of reaction mixture of the following composition: benzoyl-L-arginine ethyl ester, 13·7 mM; NaCl, M; ethylenediaminetetra-acetic acid (EDTA; mM). Appropriate volumes of enzyme are included in 15 ml. of reaction mixture. The rate of hydrolysis can be expressed as moles of acid produced/sec./mole of enzyme.

For the measurement of amide hydrolysis it was found that the photometric ninhydrin method of Moore & Stein (1954) gave sufficient sensitivity and accuracy to allow the determination of initial rates when only small quantities of ammonia had been produced.

Materials

N-Benzoyl-L-arginine ethyl ester (BAEE) and *N*-benzoyl-L-arginine amide (BAA) were prepared from L-arginine monohydrochloride (British Drug Houses Ltd.) after the method of Bergmann, Fruton & Pollock (1939).

BAA gave the following figures on analysis [the figures in parentheses are those calculated for the formula BAA, HCl,H_2O (*M*, 332)]: N = 20·4% (21·1), Cl = 9·8% (10·7), H_2O = 5·8% (5·7).

BAEE is a hygroscopic substance associated with varying amounts of water. Solutions of this substrate were analysed by trypsin hydrolysis. Assays were generally 95% of the value estimated from the known weight of substrate and a molecular weight of 343, based on the formula BAEE,HCl. The chief impurity was water.

N-Benzoylglycine methyl ester (hippuric methyl ester) was prepared from hippuric acid (British Drug Houses Ltd., London) by the ion-exchange method. IR-120 (H) resin (British Drug Houses Ltd., London) was freed from colouring matter by refluxing with methanol before use. Dried hippuric acid (5 g.) was suspended in 200 ml. of dry methanol and refluxed for 12 hr. with 5 g. of the resin. Finally the resin was filtered off and the methanol removed under vacuum. The ester was recrystallized from aqueous methanol in approx. 80% yield. This method is unsuitable for positively charged amino acid derivatives since the resin is positively charged.

Hippuric amide was prepared by saturating a solution containing 5 g. of hippuric methyl ester in 200 ml. of dry methanol with ammonia gas under anhydrous conditions. After storage of the resulting solution at room temperature for 2 days methanol was removed under vacuum until crystallization commenced. The amide was recrystallized from aqueous methanol with approx. 75% yield.

Ficin was obtained as the dried latex from L. Light and Co. Ltd., Colnbrook, Bucks. In this form the enzyme was found to be stable for periods of over 1 year, judged by the constant specific activity obtained with different preparations during this time interval. The preparation was done in a cold room at 4°. Crude latex (20 g.) was suspended in 200 ml. of 0·01 N-HCl (resulting pH 4·0) and stirred for 2 hr. The resulting suspension was then dialysed with stirring against 5 l. of mM-EDTA (pH 4·4) for 15 hr. The insoluble material was removed by centrifuging and the resultant enzyme solution [(1) of Table 1] was purified by ammonium sulphate fractionation, details of which are shown in Table 1. After each precipitation the suspension of precipitate was dialysed against mM-EDTA (pH 4·4) and the volumes indicated are those of the material redissolved in this manner. Except for pilot experiments fraction (8) was used for kinetic measurements.

The protein concentrations given in Table 1 were determined by measurement of extinction at 279 mμ after a 50-fold dilution of the enzyme solution. A calibration curve relating extinction to Kjeldahl nitrogen enabled the easy evaluation of protein concentration, by assuming that the protein contained 16·0% of Kjeldahl nitrogen. An extinction of 1 corresponded to protein concentration of 2·2%. The specific enzymic activities listed in column four have been converted into maximum velocities of hydrolysis for 0·1 ml. of a 1% protein solution, with the value of K_m for BAEE determined later.

In order to obtain ficin preparations which were fully active without the addition of a reducing substance, it was found necessary to work as rapidly as possible at low temperature (4°). Such preparations were required for work with organic mercury inhibitors and for kinetic investigations in alkaline solution.

RESULTS

Fully active ficin preparations in the absence of the activator show stability in the range pH 3·5–9·0. This is based on the immediate recovery of full activity at the pH optimum after storage at the extremes of pH for times of up to 60 min. at 25°. Fully active preparations show no enhancement of activity on the addition of 2 mM-2:3-dimercaptopropanol when first prepared, but slowly lose activity on storage at 4°. After 6 weeks the activity is approximately one-half of its initial value. It is likely that such a change is caused by oxidation of thiol groups to the disulphide form, possibly catalysed by trace-metal impurities in the manner suggested by Lamfrom & Nielson (1957). This oxidation was found to be completely reversed by the addition of dimercaptopropanol.

The addition of mercury compounds to ficin solutions (fully active without reducing substances) destroys enzymic activity. The activity is, however, completely restored by 2 mM-2:3-dimercaptopropanol. This indicates that the loss of enzymic activity is not due to denaturation of the protein. By titration of ficin activity with methylmercuric hydroxide it was found that 1 mole of this inhibitor completely inactivated 26 kg. (or 1 mole) of enzyme (Bernhard & Gutfreund, 1956). These results suggest strongly that a thiol group of the enzyme is essential for its activity and that there is only one group/enzyme molecule.

Variation of substrate and substrate concentration

Ficin-catalysed hydrolyses of BAEE, BAA and hippuric methyl ester were found to obey accurately Michaelis–Menten kinetics. The observed velocity of hydrolysis was found to be proportional to the enzyme concentration over a 25-fold range.

The main limitation of the accuracy of the Michaelis parameter determinations with Eadie (1942) plots was the maximum solubility of substrate relative to K_m. Table 2 lists the values of the

Table 1. *Assays of* N*-benzoyl-*L*-arginine ethyl ester relating to ficin preparation and description of successive operations*

Assay number and description of successive operations	Protein (%)	Total amount of protein (g.)	$10^3 \times$Specific esterase activity (moles/sec./0·1 ml. of 1% protein)
(1) Original solution (195 ml.)	2·28	4·44	4·9
(2) 0·5 Saturated ammonium sulphate precipitate (158 ml.)	1·90	3·00	5·5
(3) Supernatant (300 ml.)	0·47	1·41	0·84
(4) 0·3 Saturated ammonium sulphate precipitate (120 ml.)	1·42	1·71	9·0
(5) Supernatant (158 ml.)	0·69	1·09	2·3
(6) 0·5 Saturated sodium chloride precipitate (100 ml.)	0·81	0·81	9·7
(7) Supernatant (116 ml.)	0·75	0·87	5·0
(8) 0·5 Saturated sodium chloride precipitate (100 ml.)	0·49	0·49	9·7
(9) Supernatant (98 ml.)	0·31	0·30	8·3

Table 2. *Michaelis parameters for the ficin-catalysed hydrolyses of* N*-benzoyl-*L*-arginine ethyl ester, N-benzoyl-*L*-arginine amide, hippuric methyl ester and hippuric amide*

Results were obtained at 25° and pH 6·06 in the presence of mM-EDTA buffer and M-NaCl. For BAEE under these conditions $V_{max.} = 9{\cdot}6 \times 10^{-8}$ moles/sec./0·1 ml. of 1% enzyme solution.

Substrate	BAEE	BAA	Hippuric methyl ester	Hippuric amide
$10^2 \times$Concentration range (M)	0·5–5·0	1·0–8·0	0·25–3·25	0·40–3·0
$10^2 K_m$ (M)	2·5±0·3	4·8±0·5	4·8±1·0	13±3
$V_{max.}$ (relative values)	1·0±0·1	0·9±0·1	1·4±0·2	0·04±0·01

Michaelis parameters of the various substrates at pH 6·06 and 25°. The table also includes the range of substrate concentrations used for each determination.

Effect of pH

The stability of ficin under different conditions of pH and activator concentration has already been mentioned. Fig. 1 shows the pH-dependence of the observed rates of hydrolysis of four substrates.

For the esterase runs at low pH, where the products were incompletely ionized, a small percentage of the product did not produce hydrogen ions. Such hydrolysis rates were corrected by using pK values for benzoyl-L-arginine or benzoyl-L-glycine.

Only esterase runs were attempted in alkaline solution because of the difficulties of working in a carbon dioxide-free atmosphere. All measurements at alkaline pH were performed under an atmosphere of carbon dioxide-free nitrogen. A blank run was performed which furnished a correction for the spontaneous hydrolysis of the substrate, with possibly a small contribution from absorption of carbon dioxide. Such corrections were only a few per cent of the measured enzymic rates.

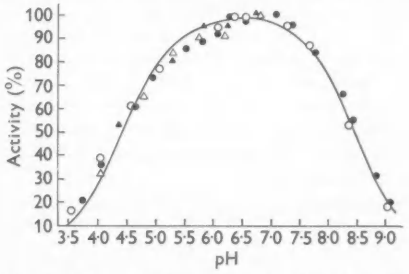


Fig. 1. Variation of observed rates of ficin-catalysed hydrolysis with pH for the substrates ○, BAEE, ●, hippuric methyl ester, △, BAA and ▲, hippuric amide. Substrate, 0·02 M; NaCl, 0·01 M. Results are expressed as percentages of their respective rates at pH 6·5, so that they can be presented as one graph.

Table 3. pH-*Dependence of* K_m *values for the action of ficin on* N-*benzoyl*-L-*arginine ethyl ester and* N-*benzoyl*-L-*arginine amide*

Results were obtained at 25° in mM-EDTA and 0·1 M-NaCl.

Substrate	pH	$10^2 K_m$ (M)
BAEE	8·43	2·1
	6·33	2·5
	6·04	2·8
	6·04	2·4
	6·04	2·6
	6·04	2·7
	6·04	2·3
	5·34	2·0
	4·63	2·3
	4·63	2·3
	4·63	2·2
	4·04	3·0
	4·04	3·3
BAA	6·30	5·2
	6·08	4·4
	5·15	4·1
	4·33	4·5

The full line in Fig. 1 is the theoretical curve for the ionization of two groups of the enzyme (pK_B 4·40 and pK_A 8·46) and indicates how closely the experimental points fit an ionization curve.

K_m measurements at 25° for the substrates BAEE and BAA at the pH specified are given in Table 3. This is to indicate the accuracy of the measurements. There may be a slight increase in K_m at pH 4·04, but in view of the reproducibility of the measurements this is barely significant.

The variation of ficin activity with pH must be interpreted in terms of ionizations of two groups on the enzyme, as the substrates used do not change their states of ionization in this pH range.

The relevant equation for the variation of enzymic activity in terms of the ionization of both a basic and an acidic group is

$$\frac{V^0}{V^1} = 1 + \frac{[H^+]}{K_B} + \frac{K_A}{[H^+]},$$

V^0 is the rate of hydrolysis at the optimum pH and V^1 is the rate of hydrolysis at other pH values. The subscripts of the ionization constants, K_A and K_B, indicate whether the ionizing group is a Brönsted acid or base at the pH optimum. The K_A term is dominant at low concentrations of hydrogen ion and the K_B term is dominant at high concentrations of hydrogen ion.

Fig. 2 shows the data of Fig. 1 for BAEE and hippuric methyl ester plotted in terms of the above equation. In acid solution the graph of V^0/V^1 versus $[H^+]$ has a slope $1/K_B$ and in alkaline solution the graph of V^0/V^1 versus $1/[H^+]$ has a slope K_A. The two pK values for each substrate at 25° are shown on the graph.

Influence of ionic strength

Ficin action has been found to depend markedly upon salt concentration. Fig. 3 shows the variation of the observed velocity of hydrolysis at low ionic strengths for the substrates BAEE, BAA and hippuric methyl ester. Different salts were used to investigate whether the effect was due to ionic strength.

It was not possible to work at ionic strengths below 0·01 M (the substrate concentration employed) because the arginine substrates themselves make a contribution to the ionic strength. It was not possible to decide whether such an effect was due to changes in one of the Michaelis parameters or in both.

At high ionic strengths, however, the results of Table 4 suggest that any change in the observed velocity of hydrolysis is due to changes in $V_{max.}$ and that K_m is almost constant. Fig. 4 presents the results of an investigation on the effects of different salts on the hydrolysis of BAEE at high ionic strengths.

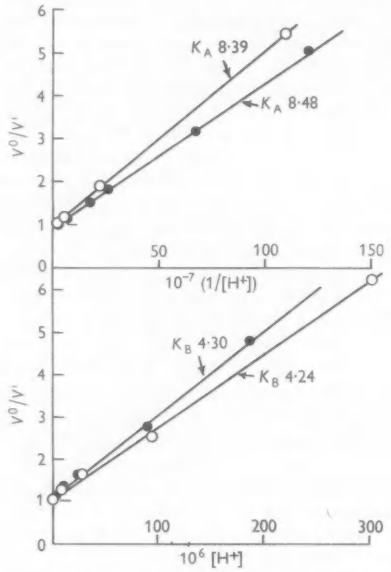


Fig. 2. Determination of the dissociation constants, K_A and K_B, of groups on the enzyme from the data of Fig. 1 and Table 3 for the pH dependence of the hydrolysis of BAEE (●) and hippuric methyl ester (○).

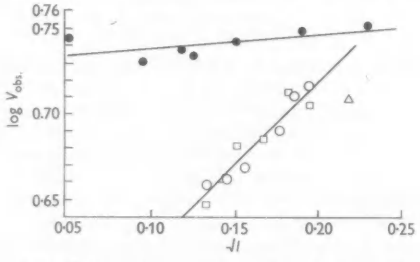


Fig. 3. Variation of observed velocity of ficin-catalysed hydrolysis of BAEE, BAA and hippuric methyl ester at low ionic strengths; pH 6·04, mM-EDTA, 0·01 M-substrate. BAEE–NaCl, ○; BAA–NaCl, △; hippuric methyl ester–NaCl, ●; BAEE–Na_2SO_4, □.

Influence of propan-2-ol

The effect of propan-2-ol on the ficin-catalysed hydrolysis of BAEE, BAA and hippuric methyl ester was studied at pH 6·3 and 25·0° with mM-EDTA as buffer and in the absence of added sodium chloride. The maximum volume concentration of propan-2-ol used was 33 % (approx. 4·3 M) and no denaturation of enzyme occurred under these conditions, as judged by the complete recovery of enzymic activity on dilution.

The results obtained for the inhibition of ficin action by propan-2-ol do not fit the usual equations for competitive or non-competitive inhibition. The variation of the Michaelis parameters for BAEE, BAA and hippuric methyl ester with propan-2-ol concentration is given in Table 5.

For all substrates the presence of propan-2-ol causes a marked increase in K_m. The influence of propan-2-ol concentration on $V_{max.}$ for the charged substrates BAEE and BAA is also very similar. For the uncharged substrate hippuric methyl ester, however, there does not appear to be any significant change in $V_{max.}$ with increasing

Table 4. *Effect of sodium chloride concentration on K_m and $V_{max.}$ for the action of ficin on* N-*benzoyl*-L-*arginine ethyl ester*

Results were obtained at 25° and pH 6·04 in mM-EDTA. In the presence of M-NaCl, $V_{max.} = 9{\cdot}6 \times 10^{-8}$ M/sec./0·1 ml. of % ficin.

[NaCl] (M)	$10^2 K_m$ (M)	$V_{max.}$ (relative) values
0·05	2·5	1·0
0·50	2·5	1·1
1·00	2·7	1·
1·70	2·8	1·5

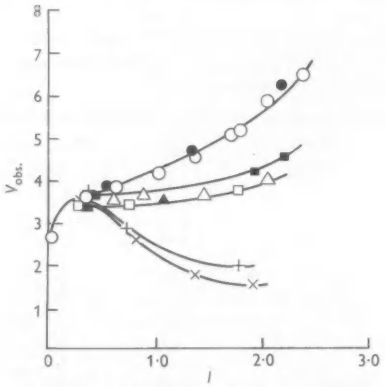


Fig. 4. Effect of different salts on the ficin-catalysed hydrolysis of BAEE; pH 6·04, mM-EDTA, 0·01 M-substrate. NaCl, ○; KCl, ●; NH_4Cl, □; KBr, ■; KI, ×; $NaNO_3$, △; KNO_3, ▲; KSCN, +.

Table 5. *Effect of propan-2-ol concentration on K_m and $V_{max.}$ for the action of ficin on N-benzoyl-L-arginine ethyl ester, N-benzoyl-L-arginine amide and hippuric methyl ester at specified* pH *and* 25°

mM-EDTA buffer was used, in the absence of added sodium chloride.

Substrate and pH	Concn. of propan-2-ol (M)	10^2 K_m (M)	$V_{max.}$ (relative) (%)
BAEE, 6·04	0	2·5	100
	0·87	4·6	76
	1·74	6·5	54
	2·63	6·7	29
BAA, 6·30	0	5	100
	1·31	11	68
	2·62	45	23
Hippuric methyl ester, 6·34	0	5	100
	0·87	9	100
	2·62	25	100

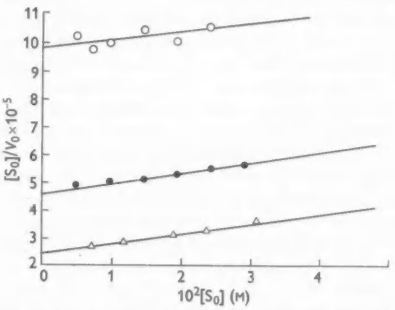


Fig. 5. Effect of propan-2-ol concentration (○, 2·62 M; ●, 0·87 M) on the ficin-catalysed hydrolysis of hippuric methyl ester at pH 6·04 in mM-EDTA. △, No propan-2-ol.

propan-2-ol concentration. The evidence for this is shown in Fig. 5, where Eadie (1942) plots for the determination of the Michaelis parameters at specified propan-2-ol concentrations are shown. The slope of an Eadie plot is $1/V_{max.}$ and the ordinate intercept is $K_m/V_{max.}$.

It is difficult to evaluate the Michaelis parameters accurately in this case because the solubility of hippuric methyl ester is low compared with the value of K_m to be determined. A decrease in $V_{max.}$ with increasing propan-2-ol concentration would require an increase in slope of the Eadie plots. The experimental results do not support this. The lines drawn through the experimental points in Fig. 5 have a constant slope which would imply that $V_{max.}$ is unaffected by propan-2-ol for this substrate. The degree to which the experimental results support this proposal may be gauged from the Figure. This relative invariance of $V_{max.}$ for hippuric methyl ester may be compared with the change in $V_{max.}$ observed with BAEE and BAA at corresponding propan-2-ol concentrations (Table 5). There is, however, an increase in K_m for hippuric methyl ester and this is illustrated by the increase in the ordinate intercept in Fig. 5 with increasing propan-2-ol concentration.

Effect of temperature

The variation of the Michaelis parameters K_m and $V_{max.}$, in the temperature range 10–40°, has been investigated for the substrates BAEE, BAA and hippuric methyl ester. The ester substrates were studied at two pH values; the amide was studied in the region of the pH optimum only. The results are set out in Table 6.

Table 6. *Variation of the Michaelis parameters with temperature and the derived Arrhenius activation energy*

mM-EDTA was used as buffer throughout. Different convenient amounts of enzyme were used for each substrate: BAEE experiments, $13·3 \times 10^{-8}$ moles of enzyme; BAA experiments, $11·5 \times 10^{-8}$ moles of enzyme; hippuric methyl ester (HME) experiments, $5·06 \times 10^{-8}$ moles of enzyme.

Substrate and pH	Temp. (° K)	$V_{max.}$ (arbitrary units)	E_a (kcal./mole)	10^2 K_m (M)
BAEE, 6·25	285·0	11·6	—	1·9
	298·0	33·2	12·8	2·5
	312·5	85·6	—	3·3
BAEE, 4·60	285·0	6·6	—	—
	298·0	19·6	14·0	—
	312·5	58·8	—	—
HME, 6·25	287·7	9·0	—	3·9
	297·9	17·7	10·4	4·8
	312·8	38·6	—	6·5
HME, 4·25	287·7	5·2	—	—
	297·9	9·1	11·0	—
	312·8	24·0	—	—
BAA, 6·25	284·2	9·1	—	4·3
	298·6	25·8	11·6	4·8
	313·7	62·4	—	5·5

Fig. 6 is a graph of the data for hippuric methyl ester to determine the activation energy, E_a, by means of the usual Arrhenius equation

$$\log k = \frac{-E_a}{2{\cdot}303\,RT} + \text{constant}.$$

This relationship was found to be linear for all substrates in the temperature range 10–40°, within experimental error.

DISCUSSION

The results of detailed kinetic investigations of the ficin-catalysed hydrolysis of a number of esters and amides give information about the physicochemical properties of those groups on the enzyme molecule which can be called the catalytic groups. From these properties one can make deductions about the probable identity of these groups and about the mechanism of the reactions which they undergo with the substrates.

A number of different methods have been used to determine the dissociation constants of groups essential for the reactivity of enzyme–substrate compounds. Our previous studies on trypsin (Gutfreund, 1955*a*) and chymotrypsin (Hammond & Gutfreund, 1955) encouraged us to use the simple method of interpreting plots of reaction rate against pH as titration curves to get a first picture of the situation. Fig. 1 shows that one acidic and one basic group are involved in the reactivity of ficin. The pK values for the two groups are evaluated from the plots in Fig. 2. Comparisons of pK values obtained for groups on protein surfaces with known constants for free amino acids have to be treated with caution. For the characterization of active groups one must consider both the general variations of pK values with charge configuration and dielectric properties of the surroundings and the special circumstances which are responsible for the peculiar reactivity of groups on the active site; this point is elaborated below. The acidic group is most likely to be a carboxyl. The basic group cannot be identified from its pK alone, but it will be shown below that there is good evidence for a positively charged group on the active site. It can therefore be concluded that an NH_3^+ group is involved. None of the substrates used has groups which change their ionization over the pH range examined here.

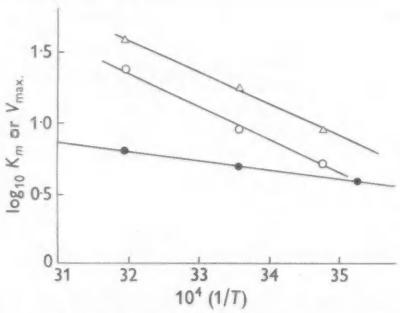


Fig. 6. Analysis of data for temperature-dependence of the ficin-catalysed hydrolysis of hippuric methyl ester (see Table 6); mM-EDTA; M-NaCl; ●, K_m at pH 6·25; △, $V_{max.}$ at pH 6·25 and ○, $V_{max.}$ at pH 4·25.

It has been shown previously (Bernhard & Gutfreund, 1956) that a sulphydryl group is required for the activity of the enzyme. This has been confirmed during the present studies by titration with methylmercuric hydroxide and subsequent reactivation by dimercaptopropanol.

Fig. 3 shows that the effect of variation of ionic strength (I) in the observed velocity of hydrolysis at low values of I is different for charged and uncharged substrates. The line drawn through the experimental points for BAEE and BAA in Fig. 3 has the theoretical slope for a uni-univalent-like charge interaction with the well-known Debye–Hückel–Brönsted equation. As the arginine substrates have unit positive charge at pH 6·0 these results suggest that there is a positively charged group at the active site of the enzyme. There is a similar enhancement of observed velocity of hydrolysis for charged and uncharged substrates at concentrations of sodium chloride greater than approximately 0·15 M.

Propan-2-ol is a suitable solvent for studies of dielectric effects on enzyme systems. Although a large variety of complex changes can occur with altered solvent composition, the data presented in Figs. 5 and 7 are readily interpreted as a dielectric effect on the charge interaction in the enzyme–substrate compound. Propan-2-ol molecules are no doubt adsorbed on the enzyme by van der Waals' forces and hydrogen bonds. This would hinder the adsorption of the substrate at the active site and the formation of the Michaelis–Menten complex. It is possible to explain the increased K_m values in the presence of propan-2-ol in this way. On a molar basis propan-2-ol is a better inhibitor than ethanol, presumably because of its greater van der Waals' attraction to the protein. The invariance of $V_{max.}$ with propan-2-ol concentration for the uncharged substrate hippuric methyl ester compared with the decrease observed for the charged substrates BAEE and BAA suggests that the last-mentioned effect is most likely due to the repulsion between the two positive charges on the substrate and active site. The variation of $V_{max.}$ for BAEE hydrolysis with propan-2-ol concentration is presented in Fig. 7 in terms of the theoretical relationship between the log of the rate constant

and the reciprocal of the dielectric constant of the medium (Scatchard, 1932). A linear relationship is obtained and its slope enables the calculation of the distance of closest approach of the arginine side chain and the active site, assuming that the interaction is between two unit positive charges. The value of 1·3 Å so obtained is small but of a reasonable magnitude for the approximations involved.

Two important conclusions can be drawn from the investigations of the effect of temperature on the ficin-catalysed hydrolysis of esters and amides. First it can be seen in Table 6 that the difference between the energy of activation at a pH near the pK of the acidic group and at pH 6·25 is small. One can derive from this that the heat of ionization of the acidic group is small (Gutfreund, 1955*a*), which would confirm that a carboxyl ionization is involved. The second point of interest is demonstrated by the fact that the energies of activation for the ficin-catalysed hydrolysis of esters and amides are very similar. [For the trypsin-catalysed hydrolysis of the same pair of ester and amide substrates considerably different energies of activation were found (Schwert & Eisenberg, 1949; Butler, 1941).] This confirms an earlier conclusion from similarity of rate of hydrolysis (Bernhard & Gutfreund, 1956) that the rate-determining step for the two substrates is the same. Since esters and amides have such widely different characteristics towards hydrolytic attack as demonstrated, for example, by the oxygen-exchange experiments of Bender, Gringer & Kemp (1954), it can only be concluded that the rate-determining step is the decomposition of an intermediate common to the reaction of ficin with BAEE and BAA. It seems reasonable to suggest that this common intermediate is an enzyme acylated by the substrate at the sulphydryl group. The reaction path of ficin (E) with BAA or BAEE (AB) can then be written:

$$E + AB \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EAB \xrightarrow{k_2} EA + B \xrightarrow{k_3} E + A + B$$

where EAB is a rapidly formed loose enzyme–substrate compound; the formation of the acyl enzyme EA and its decomposition to free enzyme and acid can be regarded as irreversible while product concentrations are low. From the discussion above it is concluded that the step characterized by k_3 is rate-determining and corresponds to the turnover number or $V_{max.}$ of the enzyme. The step characterized by k_2 (the acylation of the enzyme) must be slower for an amide substrate than for an ester. It was stated above that the similarity of overall rate of hydrolysis and energies of activation of the reactions with BAA and BAEE indicates the existence of a common intermediate. The coincidence of the dependence of the rate of reaction of the two substrates on pH, temperature and ionic strength is, however, not sufficiently exact to exclude the possibility that for BAA k_2 and k_3 are of the same order of magnitude. Some comments must be made on the relative rates of hydrolysis of hippuric methyl ester and hippuric amide. The fact that $V_{max.}$ for hippuric methyl ester is somewhat larger than for BAEE indicates that the decomposition of hippuryl acylated enzyme is somewhat faster than that of the benzoyl-L-arginyl acylated enzyme. The very much slower rate of hydrolysis of hippuric amide indicates that for this substrate the formation of the acyl enzyme is rate-determining. The low solubility of the uncharged substrates and the large K_m for hippuric amide make accurate calculation of constants for this substrate difficult.

One of us (Gutfreund, 1955*b*) has attempted to determine k_1 for the reaction of ficin with BAEE by the 'initial acceleration' method. The value of 500 M^{-1} sec.$^{-1}$ was assigned to k_1 because it was shown to be a second-order constant and only the formation of the first enzyme–substrate compound is a second-order process. It can, however, be shown from the analysis of the three-step process used to describe the reaction of this and other hydrolytic enzymes (Gutfreund & Sturtevant, 1956) that if, at substrate saturation, [EAB] $\ll$ [EA] the rate of formation of EA (the rate-determining enzyme–substrate compound) will be

$$\frac{d[EA]}{dt} = k_2[EAB],$$

and if the steady-state concentration of [EAB], which is very small compared with the total enzyme concentration, is reached rapidly, then the pseudo-second-order constant quoted above is an evaluation of k_2 [EAB].

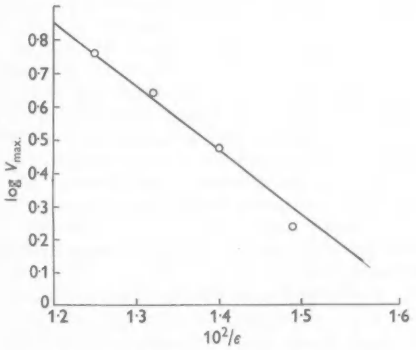
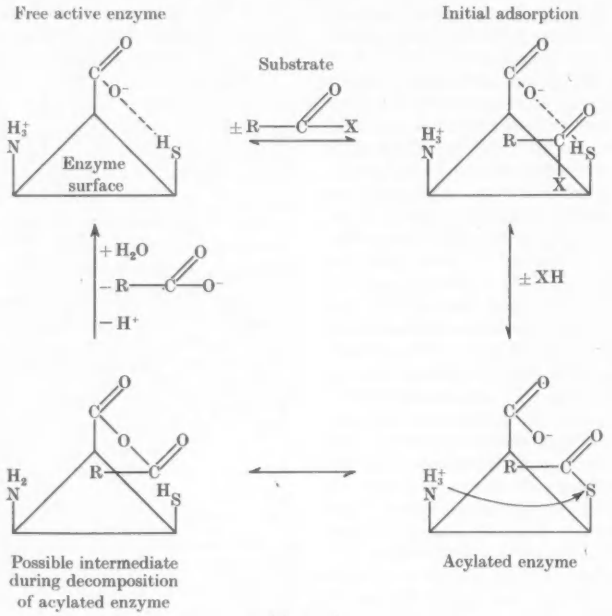


Fig. 7. Variation of log $V_{max.}$ with dielectric constant (ϵ) for the ficin-catalysed hydrolysis of BAEE (pH 6·04 in mM-EDTA (see Table 5).



Scheme 1

We have in this manner obtained good evidence for the chemical nature of the catalytic site and for the relative rates of the three distinguishable steps in the formation and decomposition of the enzyme–substrate compounds of ficin. The reaction mechanism of steps involved in the interaction, between the three groups CO_2^-, NH_3^+ and SH on the enzyme surface and the carboxyl group of different substrates, is open to a number of interesting speculations. We should like to propose the steps shown in Scheme 1 for the reaction between enzyme and substrate.

Bender (1957) has described some intramolecular-catalysis reactions which would simulate such steps in enzymic reactions. Stockell & Smith (1957), Smith, Chavré & Parker (1958) and Kimmel & Smith (1957), who have undertaken a detailed analysis of the reactions and properties of the closely similar enzyme papain, have proposed a somewhat different reaction mechanism. They assign the pK 8·4 to the catalytic –SH group and assume that the enzyme cannot be acylated by the substrate when the sulphydryl group is ionized. For ficin we object to this interpretation on two grounds. First, slowing down of the acylation by partial ionization of –SH should not affect the overall rate since the hydrolysis of the acyl enzyme is rate-determining. Secondly, this mechanism does not involve the positive group known to participate in the reaction. Our interpretation involves both ionized $-CO_2^-$ and NH_3^+ groups in the rate-determining decomposition of the acyl enzyme.

Finally it should be pointed out that the relative stability of the acyl enzyme compound accounts for the efficiency of the ficin- and papain-catalysed acyl transfer to acceptors other than water, as in transpeptidation. This is discussed by one of us elsewhere (Gutfreund, 1957) in quantitative terms.

SUMMARY

1. The kinetics of ficin-catalysed hydrolyses of benzoyl-L-arginine ethyl ester, methyl hippurate and of the corresponding amides was studied under a variety of conditions of temperature, pH, ionic strength and dielectric constant.

2. From the results of these experiments it can be concluded that three reactive groups are necessary for the catalytic action of ficin; these are –SH, NH_3^+ and CO_2^-.

3. A reaction sequence between enzyme and substrate is proposed which involves the rapid formation of a loose enzyme–substrate compound, a subsequent acylation of the enzymic –SH group by the carbonyl of the substrate and finally the decomposition of the acyl enzyme.

4. Reaction mechanisms for the second and third step can be formulated by analogy with homogeneous bifunctional catalysis.

One of us (B.R.H.) is indebted to the Ramsay Memorial Trustees for the award of a Fellowship. We should also like to record our appreciation of the most helpful and stimulating advice we have received from Dr E. L. Smith at various times.

REFERENCES

Bender, M. L. (1957). *J. Amer. chem. Soc.* **79**, 1258.
Bender, M. L., Gringer, R. D. & Kemp, K. C. (1954). *J. Amer. chem. Soc.* **76**, 3350.
Bergmann, M., Fruton, J. S. & Pollock, H. (1939). *J. biol. Chem.* **127**, 643.
Bernhard, S. A. & Gutfreund, H. (1956). *Biochem. J.* **63**, 61.
Butler, J. A. V. (1941). *J. Amer. chem. Soc.* **63**, 2971.
Eadie, G. S. (1942). *J. biol. Chem.* **146**, 85.
Gladner, J. A. & Laki, K. (1958). *J. Amer. chem. Soc.* **80**, 1263.
Gutfreund, H. (1955*a*). *Trans. Faraday Soc.* **51**, 441.
Gutfreund, H. (1955*b*). *Disc. Faraday Soc.* **20**, 167.
Gutfreund, H. (1957). *Advanc. Catalys.* **9**, 284.
Gutfreund, H. & Sturtevant, J. M. (1956). *Proc. nat. Acad. Sci., Wash.*, **42**, 719.
Hammond, B. R. & Gutfreund, H. (1955). *Biochem. J.* **61**, 187.
Kimmel, J. R. & Smith, E. L. (1957). *Advanc. Enzymol.* **19**, 267.
Koshland, D. E. & Erwin, M. J. (1957). *J. Amer. chem. Soc.* **79**, 2657.
Lamfrom, H. & Nielson, S. O. (1957). *J. Amer. chem. Soc.* **79**, 1966.
Moore, S. & Stein, W. H. (1954). *J. biol. Chem.* **211**, 907.
Scatchard, G. (1932). *Chem. Rev.* **10**, 229.
Schwert, G. S. & Eisenberg, M. A. (1949). *J. biol. Chem.* **179**, 665.
Smith, E. L., Chavré, V. J. & Parker, M. J. (1958). *J. biol. Chem.* **230**, 283.
Stockell, A. & Smith, E. L. (1957). *J. biol. Chem.* **227**, 1.
Wilson, I. B. & Calib, E. (1956). *J. Amer. chem. Soc.* **78**, 202.

The Effects of Adenosine Triphosphate on the Mechanical Properties of Surface Films of L-Myosin and Actomyosin

By D. F. CHEESMAN, M. MARIE KEELER* and O. STEN-KNUDSEN†
Department of Physiology, Bedford College, University of London

(*Received* 20 *October* 1958)

The earliest study of the effects of the adenine nucleotides on the properties of the myofibrillar proteins spread at the air–water interface was made by Munch-Petersen (1948), who found that when actomyosin was spread on 0·5 M-potassium chloride solution containing 0·1 mM-adenosine triphosphate, it occupied an area considerably greater, for a given surface pressure, than in the absence of adenosine triphosphate. The effect was later shown to be due, at least in part, to an increase by adenosine triphosphate in the rate of spreading of the protein (Lajtha & Rideal, 1952; Cheesman, 1952). Similar effects were found for actin-free myosin by Munch-Petersen, although Cheesman was unable to repeat this observation.

The acceleration by adenosine triphosphate of the spreading of actomyosin may be explained by the dissociation of the protein by this nucleotide into its constituent actin and myosin. A similar acceleration is produced by high salt concentrations, which also dissociate actomyosin in bulk solution. For this reason, the results seemed to be of little biochemical interest, especially as no evidence of chemical alteration of the protein by adenosine triphosphate could be obtained from phase-boundary potential measurements (Cheesman, 1952).

Cheesman & Sten-Knudsen (1959) have developed a procedure for the study of the viscoelastic properties of protein films. It was found with this method that when actomyosin or L-myosin was spread on M-potassium chloride solution containing 0·1 mM-adenosine triphosphate, the mechanical rigidity of the resulting film was considerably lower than that of a similar film in the absence of adenosine triphosphate at the same surface pressure with the first protein, and at the same surface pressure and specific area with the second. Two explanations of these findings presented themselves. It was conceivable that a complex of adenosine triphosphate and denatured protein persisted in the spread film, with a consequent effect upon the mechanical properties of the latter. Alternatively, since the protein under the conditions of the experiments was not fully spread, the effect could have been due to an interaction between adenosine triphosphate and native

* Mother Marie de l'Enfant-Jésus, R.S.H.M. Present address: Marymount College, Tarrytown-on-Hudson, N.Y., U.S.A.

† Present address: Universitetets Neurofysiologiske Institut, Copenhagen, Denmark.

protein forming an integral part of the film structure. The first explanation, if it proved to be correct, would be of some general interest. The second, on the other hand, would offer considerable possibilities in the study of the interaction of adenosine triphosphate and the muscle proteins, especially in relation to the much-disputed theory that the phosphorylation of actomyosin by adenosine triphosphate is an essential feature of the contraction cycle in living muscle (Weber, 1955; Morales, Botts, Blum & Hill, 1955). For this reason it was decided to undertake a more detailed study of the phenomenon, the results of which are now presented with earlier data which have not hitherto been published except in outline (Cheesman & Davies, 1954).

EXPERIMENTAL

Materials

Adenosine triphosphate (*ATP*). The material used in the earlier experiments was obtained from the Ba salt supplied by Boots Pure Drug Co. Ltd. (Nottingham) and purified by way of the Hg and Ba salts (LePage, 1949). All experiments were repeated, with similar outcome, with the crystalline disodium salt from the Sigma Chemical Co. (St Louis, Mo., U.S.A.).

Adenosine diphosphate (*ADP*). This was the Na salt from the Sigma Chemical Co.

Inosine triphosphate (*ITP*). This was prepared from ATP by the method of Kaplan (1957).

2:4-*Dinitrophenol* (*DNP*). This was of analytical quality and was recrystallized from water and used in the form of the Na salt.

Fuadin (*antimony bis-*[*catecholdisulphonic acid*], *Na salt*). This was generously provided by Farbenfabriken Bayer, Leverkusen.

Ethylenediaminetetra-acetic acid (*EDTA*). This was obtained from British Drug Houses Ltd., and *p*-chloromercuribenzoic acid (PCMB) was obtained from L. Light and Co. Ltd. Solutions of both were brought to pH 7·4 with NaOH before use.

All inorganic salts and buffer substances were of analytical grade.

The water used in all experiments was from the laboratory distilled-water supply, further purified by passage over mixed anion- and cation-exchange resins in an Elgastat deionizer (Elga Products Ltd., London, S.W. 19).

L-Myosin. This was prepared from the leg and back muscles of rabbits by the method of Mommaerts & Parrish (1951) with five reprecipitations.

Actomyosin. This was made from similar material by extraction of the minced muscle for 16 hr. at 5° with Weber's solution (0·6 M-KCl, 0·04 M-$NaHCO_3$, 0·01 M-Na_2CO_3; 300 ml./100 g. of muscle). The extract was strained through muslin and centrifuged at 1500 *g* for 20 min. Actomyosin was precipitated from the supernatant with 3 vol. of water at 5°; it was centrifuged and redissolved in 0·25 vol. of 2 M-KCl. The protein was reprecipitated by dilution with 3 vol. of water. The reprecipitation was repeated four times with occasional small additions of Weber's solution to maintain a slightly alkaline pH.

Actin. This was obtained from the muscle residue in the L-myosin preparation by the method of Straub (1942) as modified by Bailey & Perry (1947).

L-Myosin and actomyosin were kept in solution in 0·5 M-KCl at 5°. A few drops of toluene were added to delay bacterial attack. The preparations were used, unless otherwise stated, within 14 days.

Methods

Dry-weight determinations on protein solutions. These were made as follows: 5 ml. of solution was treated with 1 ml. of 10% (w/v) trichloroacetic acid in a weighed centrifuge tube. The precipitate was centrifuged, washed twice with water and once with ethanol on the centrifuge and dried to constant weight at 110°.

Determination of adenosine triphosphatase activity. The incubation mixture consisted of 1 ml. of 5 mM-ATP, 1 ml. of 0·05 M-2-amino-2-hydroxymethylpropane-1:3-diol–HCl buffer, pH 7·4 (tris–HCl buffer: Gomori, 1946) in 0·5 M-KCl and 0·05 ml. of 0·1 M-$CaCl_2$ at 37°. At zero time about 0·25 mg. of L-myosin or actomyosin in 1 ml. of 0·5 M-KCl at 37° was added. The reaction was stopped after 5 min. with 2·5 ml. of 8% (w/v) trichloroacetic acid. The precipitated protein was removed by filtration through cotton wool into a 25 ml. graduated flask. The cotton wool was washed once with 0·5 ml. of trichloroacetic acid and twice with 1 ml. of water. Inorganic phosphate in the filtrate was estimated by the method of Fiske & Subbarow (1925), the volume being made up to 25 ml. before the colorimetric reading in the Hilger Spekker absorptiometer. Reagent blanks and standards were run with all determinations.

Measurements of film plasticity. The modified Langmuir surface balance used for the study of the viscoelastic properties of the protein films was identical with that described by Cheesman & Sten-Knudsen (1959). The technique consisted in following the time course of the horizontal displacement in the film of a waxed mica float, of 6 mm. radius, bearing a saturated permanent magnet, during the application of an electric field from a solenoid. The solenoid was mounted near the trough with its axis along the surface. The force–current and force–distance relationships for solenoid and magnet were previously determined by direct measurement with the aid of a torsion balance. The experimental procedure was as follows, with occasional variations indicated where they are relevant.

Protein solution (40 μl.) in 0·5 M-KCl, containing about 0·04 mg. of actomyosin or L-myosin, was applied in small drops to the surface of the substrate in the trough. The available area was about 0·8 m.^{2}/mg. of protein. During the spreading period the magnetic float was held in position in the mid-line of the trough at about 5 cm. from the floating mica boom of the balance by means of a small solenoid mounted above the surface. Five minutes after the application of the protein, the film was compressed to a surface pressure of 3 dynes/cm. as recorded on the torsion head. The current through the small solenoid was then switched off and the large displacing solenoid was adjusted to a standard distance (usually 18·5 cm.) from the centre of the magnetic float. The zero position of the float was recorded with a telescope bearing a micrometer eyepiece. The displacing current and a stop clock were turned on exactly 7 min. after the application of the protein. The positions of the float were read at intervals of 5 sec. The displacing current was interrupted at 30 sec. and the

readings were continued for a short time thereafter. During such experiments the surface pressure usually increased by about 0·5 dyne/cm.

The displacement of the float may be resolved into components representing viscous flow and elastic deformation in the film. The presence of the latter component is manifested in elastic recovery after removal of the stress. Since the results recorded here are meant to be only semi-quantitative, displacement curves are reproduced without mathematical treatment. In most instances only the displacements of the float at 30 and 40 sec. (10 sec. after interruption of the current) are given, the former quantity being taken as a rough measure of 'plasticity' (see below), the latter as an indication of the importance of the flow component. In the legends of Figs. 1 and 2, values are given for the elastic moduli of the films as calculated by the empirical procedure described by Cheesman & Sten-Knudsen (1959).

The term 'plasticity' is employed in this paper as an antonym to 'stiffness', which in turn is used to signify viscoelastic resistance to mechanical deformation. When therefore ATP is said to have a plasticizing (or stiffening) action on a film, it is implied that this substance has reduced (or increased) the sum of the viscous and elastic forces, determined by the film structure, which resist the movement of the float under a given stress.

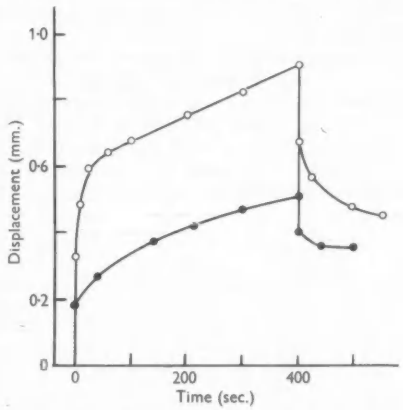


Fig. 1. Plasticizing effect of ATP on an actomyosin film. This figure, with Figs. 2, 5 and 6, shows the time course of the displacement of the magnetic float under the stress imposed by the solenoid. This displacement may be resolved into a rapid initial movement against an elastic force and a much slower drift in a viscous medium. Elastic recovery occurs when the stress is removed, in this case at 400 sec. An empirical treatment of such curves permits the calculation of elastic moduli for the films (Cheesman & Sten-Knudsen, 1959). Substrate: M-KCl, 0·01 M-veronal–HCl buffer, pH 7·0. Surface pressure: 10 dynes/cm. Specific areas: without ATP, 0·4 m.²/mg.; with ATP, 0·45 m.²/mg. Applied stress: 0·45 mg. weight, interrupted at 400 sec. ●, Without ATP (elastic modulus 5·6 dynes/cm.²); ○, with 0·1 mM-ATP (elastic modulus 3·0 dynes/cm.²).

RESULTS

Effects of adenosine triphosphate on films of L-myosin and actomyosin. The fundamental effect with which we are concerned is illustrated in Figs. 1 and 2, which represent measurements of the viscoelastic properties of films of L-myosin and actomyosin in the presence and absence of ATP. In both cases the plasticity of the film is increased by ATP.

It was observed in the early stages of the work that, after the proteins had been stored in the refrigerator for a few days, the effect of ATP upon the films decreased. Preparations of L-myosin more than 14 days old frequently showed no effect whatever. Films of aged actomyosin, on the other hand, were often stiffened by ATP. Since these aged-protein preparations had a greatly reduced adenosine triphosphatase (ATPase) activity, the matter appeared to merit further study. An experiment was made in which the enzymic activity of actomyosin was reduced progressively by incubation at 37°. Samples were withdrawn at intervals and used for film experiments which were made concurrently with estimations of ATPase activity. The results are shown in Fig. 3, where it will be seen that whereas the rigidity of the actomyosin film decreases with the reduction in the ATPase activity the presence of ATP reverses this effect, so that the plasticizing action of ATP is transformed

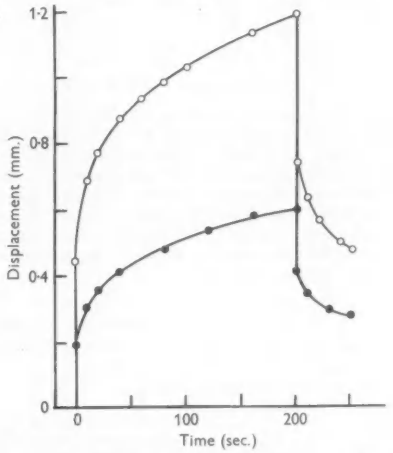


Fig. 2. Plasticizing effect of ATP on an L-myosin film (see legend to Fig. 1). Surface pressure: 15 dynes/cm. Specific area: 0·5 m.²/mg. Applied stress: 1·2 mg. weight, interrupted at 200 sec. Other conditions were as described for Fig. 1. ●, Without ATP (elastic modulus 10·6 dynes/cm.²); ○, with 0·1 mM-ATP (elastic modulus 5·1 dynes/cm.²).

into a stiffening action. The data in Table 1 show that the 'ageing' of the actomyosin corresponds to an increase in the area of the film at constant surface pressure in the absence of ATP, although this area remains unchanged in the presence of ATP.

Transience of the effect of adenosine triphosphate. At low surface pressures it is found that the plasticizing action of ATP upon films of L-myosin and actomyosin is of short duration. The data in Fig. 4, from an experiment with L-myosin, show an almost complete elimination of the effect after 15 min. The rise in surface pressure during this period suggests that increased surface denaturation is the chief contributory factor here. It is interesting to compare these data with Figs. 1 and 2, which show the persistence of the effect at higher surface pressures. These observations lend support to the view that the ATP effect is determined by the native component in the film.

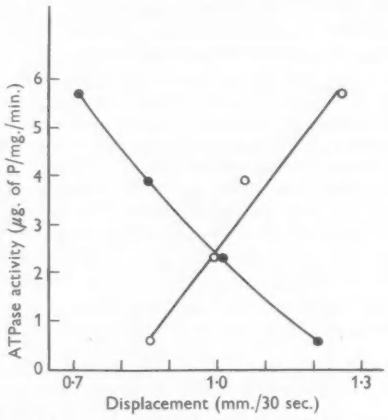


Fig. 3. Influence of pre-heating of the protein on the effect of ATP. Fresh actomyosin was heated in 0·5 M-KCl at 37°. Samples were taken at 3, 10, 20 and 30 min. for ATPase assays and for film experiments in the presence and absence of ATP. Substrate for films: 0·8 M-KCl, 0·01 M-tris–HCl buffer, pH 7·4. Spreading time: 5 min. Applied stress: 0·9 mg. weight. Displacement of float was recorded at 30 sec. ●, Without ATP; ○, with 0·1 mM-ATP.

Table 1. *Changes in adenosine triphosphatase activity and rate of spreading of actomyosin incubated at* 37°

See also Fig. 3.

Time of incubation (min.)	ATPase activity (μg. of P/mg./min.)	Area of film after 5 min. spreading and compression to 3 dynes/cm. (m.²/mg. of protein)	
		With ATP	Without ATP
3	5·7	0·58	0·53
10	3·9	0·58	0·54
20	2·4	0·58	0·55
30	0·6	0·58	0·58

Influence of p-*chloromercuribenzoate on the effect of adenosine triphosphate.* When the thiol reagent PCMB is present in the substrate, the effect of ATP on the plasticity of L-myosin and actomyosin films is entirely eliminated (Table 2). We have never found ATP to exert a stiffening effect in systems inhibited with PCMB, although the inhibitor itself gives such an effect in higher concentrations, presumably due to cross-linking over the carboxyl group by PCMB molecules which have combined with thiol groups in the protein.

Influence of 2:4-*dinitrophenol on the effect of adenosine triphosphate.* Greville & Needham (1955) and Chappell & Perry (1955) established that DNP increased the rate of liberation of inorganic phosphate from ATP by L-myosin (at all ionic strengths) and by actomyosin (at high ionic strengths). It was therefore of interest to study the effect of this substance in our systems. mM-DNP almost completely inhibits the plasticizing effect of ATP on films of L-myosin (Fig. 5). With actomyosin, the action of ATP is converted into a stiffening (Table 2).

Influence of ethylenediaminetetra-acetate on the effect of adenosine triphosphate. EDTA, which also activates the ATPase of L-myosin and actomyosin in a striking degree (Bowen & Kerwin, 1954), modifies the effect of ATP on films of the two proteins in the same sense as does DNP. With L-myosin this influence is not as marked as with DNP (Table 2).

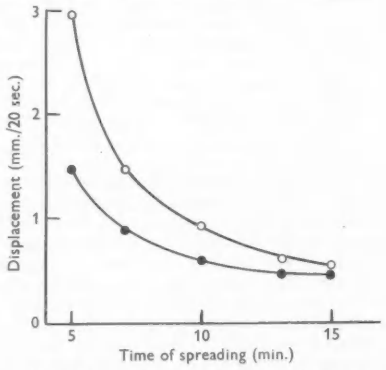


Fig. 4. Decrease with time in the effect of ATP on films of L-myosin. Substrate was as for Fig. 3. Spreading time before compression: 5 min. Surface pressure increased from 3 to 5 dynes/cm. in 15 min. Applied stress: 0·9 mg. weight. Displacements were recorded at 20 sec. ●, Without ATP; ○, with 0·1 mM-ATP.

Table 2. *Influence of various substances on the plasticizing action of adenosine triphosphate on films of L-myosin and actomyosin*

Figures represent the means of at least two experimental values. Since several protein preparations were used, and these at different times after preparation, experiments are comparable only with those in the same group. The substrate in all cases was 0·8 M-KCl buffered at pH 7·4 with 0·01 M-tris–HCl. ATP was added in a concentration of 0·1 mM; other substances, when not otherwise indicated, in concentrations of mM. Spreading time: 5 min. Surface pressure: 3 dynes/cm. Specific areas: actomyosin 0·45–0·55 m.²/mg. (increased by 10–18% with ATP); L-myosin 0·5–0·6 m.²/mg. (unaffected by ATP).

Protein	Additions to substrate	Stress (interrupted at 30 sec.) (mg.)	Displacement of float (mm.) 30 sec.	40 sec.
L-Myosin	None	0·4	0·57	0·43
	PCMB		0·37	0·28
	ATP		1·07	0·61
	ATP + PCMB		0·46	0·37
Actomyosin	None	1·2	1·34	0·75
	ATP		2·09	1·31
	ATP + PCMB (0·01 mM)		1·27	0·60
Actomyosin	None	0·9	1·16	0·62
	DNP		1·17	0·62
	ATP		1·42	0·73
	ATP + DNP		0·72	0·31
L-Myosin	None	0·9	1·34	0·76
	EDTA		1·37	0·78
	ATP		2·12	1·48
	ATP + EDTA		1·78	0·98
Actomyosin	None	0·4	0·69	0·42
	EDTA		0·69	0·42
	ATP		0·97	0·66
	ATP + EDTA		0·42	0·03
L-Myosin	None	0·4	0·57	0·43
	Fuadin		0·57	0·34
	ATP		1·07	0·52
	ATP + fuadin		0·64	0·47

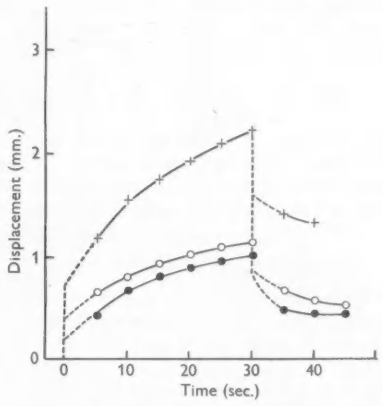


Fig. 5. Inhibition by DNP of the effect of ATP on L-myosin films (see legend to Fig. 1). Substrate was as for Fig. 3. Spreading time: 5 min. Surface pressure: 3 dynes/cm. Applied stress: 0·9 mg. weight. Stress was removed at 30 sec. ●, Protein alone or with mM-DNP; +, with 0·1 mM-ATP; ○, with 0·1 mM-ATP and mM-DNP.

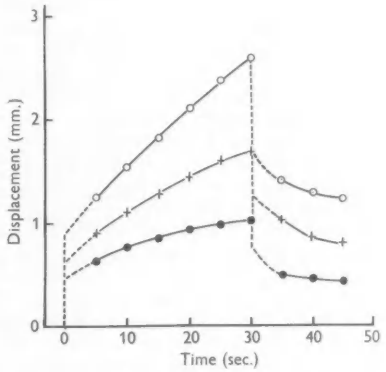


Fig. 6. Stimulation by fuadin of the effect of ATP on actomyosin films (see legend to Fig. 1). Conditions were as for Fig. 5. ●, Protein alone or with mM-fuadin; +, with 0·1 mM-ATP; ○, with 0·1 mM-ATP and mM-fuadin.

Influence of fuadin on the effect of adenosine triphosphate. Weber (1955) found that fuadin (antimony bis-[catecholdisulphonic acid], Na salt), a weak inhibitor of actomyosin ATPase, when added to a system containing adenosine [^{32}P]-diphosphate, ATP and actomyosin, made it possible to detect a transfer of phosphate groups between ATP and ADP.

This substance, when added to the substrate in our systems, had no influence on the elastic properties of L-myosin or actomyosin films. In the presence of ATP, the plasticizing action of the latter on L-myosin films was almost completely inhibited (Table 2). With actomyosin, on the other hand, the effect of ATP was greatly augmented by fuadin (Fig. 6).

Influence of calcium and magnesium ions on the effect of adenosine triphosphate. Calcium ions, added to the substrate as $CaCl_2$, have no significant effect on the elastic properties of L-myosin or actomyosin films, in the presence or absence of ATP. Magnesium ions, on the other hand, although they are without influence upon the protein films themselves, modify the effect of ATP on films of both proteins. In concentrations of 0·01 mM, $MgCl_2$ converts the plasticizing action of 0·1 mM-ATP into a stiffening action (slight with L-myosin). When, however, the $MgCl_2$ concentration is increased to mM, the plasticizing action of ATP is restored (Table 3). It cannot at present be said how far these results are determined by the presence of Mg and Ca in the protein preparations.

Specificity of the effect of adenosine triphosphate. It has been established that neither ADP nor ITP has a significant effect upon the elasticity of films of L-myosin or actomyosin in a concentration of 0·1 mM (Table 4). The effect of ATP is detectable at μM.

Table 3. *Influence of calcium and magnesium ions on the plasticizing action of adenosine triphosphate on films of L-myosin and actomyosin*

Conditions were as in Table 2.

Protein	Additions to substrate	Stress (interrupted at 30 sec.) (mg.)	Displacement of float (mm.) 30 sec.	40 sec.
L-Myosin	None	1·4	1·60	—
	$CaCl_2$ (mM)		1·71	—
	ATP		2·77	—
	ATP + $CaCl_2$		2·71	—
L-Myosin	None	1·4	1·75	0·85
	$MgCl_2$ (0·01 mM)		1·75	0·85
	$MgCl_2$ (mM)		1·75	0·85
	ATP		2·90	1·92
	ATP + $MgCl_2$ (0·01 mM)		1·48	0·75
	ATP + $MgCl_2$ (mM)		2·28	1·20
Actomyosin	None	1·4	1·34	—
	$CaCl_2$ (mM)		1·31	—
	ATP		2·77	—
	ATP + $CaCl_2$ (mM)		2·71	—
	ATP + $CaCl_2$ (0·01 mM)		2·68	—
Actomyosin	None	1·4	1·09	0·60
	ATP		1·35	0·69
	ATP + $MgCl_2$ (0·01 mM)		0·71	0·23
	ATP + $MgCl_2$ (mM)		1·23	0·67

Table 4. *Comparison of the effects of different nucleotides*

Conditions were as in Table 2. Nucleotides were added in concentrations of 0·1 mM.

Protein	Additions to substrate	Stress (interrupted at 30 sec.) (mg.)	Displacement of float (mm.) 30 sec.	40 sec.
L-Myosin	None	0·4	0·67	0·36
	ADP		0·71	0·40
	ITP		0·71	0·43
	ATP		1·27	0·72
Actomyosin	None	0·4	0·69	0·37
	ITP		0·64	0·30
	ATP		1·03	0·50

Actin films. G-Actin spreads readily at water surfaces (Lajtha & Rideal, 1952). Although we have not yet studied the properties of its films in any detail, preliminary experiments have shown that their elastic properties are unaffected by ATP in the presence or absence of magnesium.

DISCUSSION

Structure of the protein films

Actomyosin and L-myosin spread on the surfaces of salt solutions at a low rate. The experiments described in this paper were made under conditions where the area was of the order of 0·5 m.²/mg. at a surface pressure of 1 dyne/cm., or little more than half that for a protein film in which the unfolding of the polypeptide structure in the surface has proceeded to completion (cf. Bull, 1947). In protein films where the molecules are not fully unfolded, a proportion of these retain their native properties, as has been demonstrated for actomyosin by Hayashi (1952). Since native molecules of corpuscular proteins are likely to show only small surface activity, it appears to be a reasonable assumption that an incompletely spread film consists of an upper layer of unfolded molecules with an adsorbed layer of native molecules beneath it. Evidence in support of this view is presented by Cheesman & Schuller (1954). It may be conceived that the adsorbed, native component of the film, through the polar groups on the surface of its molecules, will establish cross-linkages between the unfolded polypeptide chains of the upper, denatured layer and thus contribute to the mechanical rigidity of the film as a whole (Cheesman & Sten-Knudsen, 1959). Conditions for this effect would be especially favourable with molecules possessing high axial ratios and high contents of ionizable side chains. L-Myosin and actomyosin have these characteristics in a marked degree (Bailey, 1954). The linkages between the actin and myosin components of actomyosin in the native layer might themselves contribute to the mechanical strength of the film.

Effects of adenosine triphosphate

It seems probable, in view of the above considerations, that the plasticizing action of ATP on films of actomyosin is due in part to the acceleration of the spreading of this protein by ATP. Thus under the conditions of our experiments, the area of actomyosin films was nearly 20% greater at 3 dynes/cm. surface pressure in the presence than in the absence of ATP (in a typical case, 0·55 as against 0·47 m.²/mg.), with a correspondingly lower surface concentration. Although the importance of this factor is difficult to investigate directly, it may be pointed out that substances such as DNP which, in small concentrations, greatly modify the plasticizing action of ATP on actomyosin films, are without significant effect on the rate of spreading of this protein in the presence or absence of ATP. We have not found the rate of spreading of L-myosin to be measurably altered by ATP under the conditions of our experiments.

Since the effect of ATP on the mechanical rigidity of the films is transient at low surface pressures, there can be little doubt that it reflects an interaction between the nucleotide and native-protein molecules forming part of the film structure. This effect might be due either to the presence of a protein–nucleotide complex or to that of an altered protein resulting from the decomposition of such a complex. In either event ATP, or groups introduced by ATP, could modify the viscoelastic properties of the film by competition for polar groups in the protein and alteration of the charge distribution.

The most striking difference between actomyosin and L-myosin films is that, whereas both are plasticized by ATP, this effect on actomyosin is converted into a stiffening by a variety of agents such as DNP, EDTA and preliminary ageing of the protein; the same agents tend merely to inhibit the effect of ATP on L-myosin films. Another agent, fuadin, enhances the ATP effect on actomyosin and eliminates that on L-myosin. The simplest explanation of these phenomena is that actomyosin interacts with ATP in two manners which are antagonistic in their effects on the viscoelastic properties of the film, one of which is inhibited by DNP, the other by fuadin. With L-myosin, on the other hand, there is no evidence of such antagonism: it suffices to assume a single mode of interaction with the nucleotide, the effect of which is reduced by all the agents enumerated above.

It is known from the work of Weber (1955) and Ulbrecht & Ulbrecht (1957) that actomyosin, glycerol-extracted myofibrils and such myofibrils from which most of the L-myosin has been removed catalyse an exchange of phosphate groups between ATP and adenosine [^{32}P]diphosphate activated by magnesium but not by calcium. This reaction was first detected in a medium containing fuadin, a weak inhibitor of the ATPase. It probably involves an intermediate phosphorylation of the protein which, if it occurred in actomyosin films, might well exert a plasticizing action. This would be expected to be unimpaired by fuadin and, indeed, to be enhanced if the latter substance inhibited an interaction between protein and ATP which tended to stiffen the films. Although the effect of DNP on the phosphate-exchange reaction catalysed by actomyosin has apparently not been studied, an

analogous reaction in mitochondria is inhibited by this compound (Cooper & Lehninger, 1957). It is hence conceivable that DNP inhibits the exchange reaction in actomyosin films, so that ATP has a resultant stiffening effect.

The stiffening action of ATP might be due to a charge effect or to a cross-linking by the nucleotide between components of the actomyosin complex. It becomes evident when the ATPase activity of the protein is decreased by heating. If the mechanism of the exchange reaction were simultaneously inactivated, this would in itself explain the progressive decrease in film plasticity. The outcome of the heating experiments would suggest a binding of ATP at sites not associated with enzymic activity, although it cannot be excluded that active centres will continue to bind nucleotide after their catalytic function has been lost, with a consequent increase in the stability of the protein–ATP complex. The high ionic strength used in our experiments presumably brought about a considerable dissociation of actomyosin; the effects of ATP, DNP and EDTA are hence probably due in part to the mechanisms suggested below for films of L-myosin.

The phosphate-exchange reaction is not catalysed by L-myosin and hence cannot be responsible for the plasticizing effect of ATP on this protein. An irreversible phosphorylation of the protein by ATP, such as was suggested in a preliminary communication (Cheesman & Keeler, 1958), might nevertheless be imagined. Brahms & Rzysko (1958) claim to have detected by chemical means a phosphorylation of H-meromyosin by ATP. A more probable explanation of our results, suggested to one of us by Dr J. Gergely, is that the increase in film plasticity is due to the presence of the enzyme–substrate complex and that the effects of DNP and EDTA consist of a reduction in the steady-state concentration of that complex. Fuadin, as an ATPase inhibitor, might be expected to have the same effect as PCMB in blocking the formation of the complex, thus inhibiting the ATP effect. This is indeed observed.

Further evidence for this view may be found in the failure of ITP to plasticize films of L-myosin in a concentration of 0·1 mM. It is difficult to suggest a plausible mechanism whereby the splitting of ATP by L-myosin, but not that of ITP, would result in phosphorylation of the protein. Kielley, Kalckar & Bradley (1956) have shown, however, that the Michaelis–Menten constant (K_m) for the ATPase reaction of calcium-activated L-myosin is much lower than that for the inosine triphosphatase (ITPase) reaction ($< 3 \times 10^{-5}$ as against $1{\cdot}9 \times 10^{-4}$) whereas the ITPase activity is some 4·5 times greater in the presence of mM-substrate. Furthermore, the ATPase reaction for L-myosin activated with mM-EDTA and 0·4 M-KCl gave K_m $2{\cdot}9 \times 10^{-4}$ and an activity about three times that for the calcium-activated enzyme. The steady-state concentration of enzyme–substrate complex in our experiments would hence be expected on the basis of simple Michaelis–Menten theory to be very much lower for ITP than for ATP at nucleotide concentrations of 0·1 mM. Similarly, EDTA might reasonably be supposed to reduce the concentration in the films of the L-myosin–ATP complex.

A puzzling feature of our results is the failure of magnesium and calcium ions in mM-concentration to affect significantly the plasticizing action of ATP, whereas the former have an inhibitory action at 0·01 mM. If the phosphate-exchange reaction contributes to the effect of ATP on actomyosin, it might be expected that calcium would remove this contribution. The behaviour of actomyosin and L-myosin towards bivalent cations in a heterogeneous system at high ionic strength is, however, unknown and cannot readily be predicted.

SUMMARY

1. Films of L-myosin and actomyosin at the air–water interface, when incompletely spread, incorporate native-protein molecules which probably contribute to their mechanical properties.

2. When such films are spread on potassium chloride solutions containing adenosine triphosphate (0·1 mM), the latter causes a marked decrease in their mechanical strength. This effect is not given by adenosine diphosphate or inosine triphosphate in the same concentration and is inhibited by *p*-chloromercuribenzoate.

3. With L-myosin, the effect is reduced or eliminated by 2:4-dinitrophenol, ethylenediaminetetra-acetate and fuadin.

4. With actomyosin the effect is enhanced by fuadin, and it is converted into a stiffening action by 2:4-dinitrophenol and ethylenediaminetetra-acetate.

5. Calcium ions do not modify the effect of adenosine triphosphate on either protein. Magnesium ions (0·01 mM) produce stiffening of both types of films in the presence of adenosine triphosphate, but the plasticizing effect reappears with higher concentrations of these ions (mM).

6. Aged or heated actomyosin solutions yield films which are stiffened by adenosine triphosphate.

7. It is concluded that adenosine triphosphate interacts with actomyosin films in at least two manners, one of which may involve phosphorylation of the protein. The effect of adenosine triphosphate on L-myosin films may be explained by the formation of an enzyme–substrate complex.

REFERENCES

Bailey, K. (1954). In *The Proteins*, vol. 2, part B, p. 951. Ed. by Neurath, H. & Bailey, K. New York: Academic Press Inc.

Bailey, K. & Perry, S. V. (1947). *Biochim. biophys. Acta*, **1**, 506.

Bowen, W. J. & Kerwin, T. D. (1954). *J. biol. Chem.* **211**, 237.

Brahms, J. & Rzysko, Ch. (1958). *Abstr. 4th Int. Congr. Biochem., Vienna*, p. 83.

Bull, H. B. (1947). *Advanc. Protein Chem.* **3**, 95.

Chappell, J. B. & Perry, S. V. (1955). *Biochim. biophys. Acta*, **16**, 285.

Cheesman, D. F. (1952). *J. Physiol.* **116**, 14*P*.

Cheesman, D. F. & Davies, J. T. (1954). *Advanc. Protein Chem.* **9**, 439.

Cheesman, D. F. & Keeler, M. M. (1958). *Abstr. 4th Int. Congr. Biochem., Vienna*, p. 84.

Cheesman, D. F. & Schuller, H. (1954). *J. Colloid Sci.* **9**, 113.

Cheesman, D. F. & Sten-Knudsen, O. (1959). *Biochim. biophys. Acta* (in the Press).

Cooper, C. & Lehninger, A. L. (1957). *J. biol. Chem.* **224**, 561.

Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.

Gomori, G. (1946). *Proc. Soc. exp. Biol., N.Y.*, **62**, 33.

Greville, G. D. & Needham, D. M. (1955). *Biochim. biophys. Acta*, **16**, 285.

Hayashi, T. (1952). *J. gen. Physiol.* **36**, 139.

Kaplan, N. O. (1957). In *Methods in Enzymology*, vol. 3, p. 873. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Kielley, W. W., Kalckar, H. M. & Bradley, L. B. (1956). *J. biol. Chem.* **219**, 95.

Lajtha, A. & Rideal, E. K. (1952). *Arch. Biochem. Biophys.* **33**, 252.

LePage, G. (1949). In *Biochemical Preparations*, vol. 1, p. 5. Ed. by Carter, H. E. New York: John Wiley and Sons Inc.

Mommaerts, W. F. H. M. & Parrish, R. G. (1951). *J. biol. Chem.* **188**, 545.

Morales, M. F., Botts, J., Blum, J. J. & Hill, T. L. (1955). *Physiol. Rev.* **35**, 475.

Munch-Petersen, A. (1948). *Nature, Lond.*, **162**, 537.

Straub, F. B. (1942). *Stud. Inst. med. Chem. Univ. Szeged*, **2**, 3.

Ulbrecht, G. & Ulbrecht, M. (1957). *Biochim. biophys. Acta*, **25**, 100.

Weber, H. H. (1955). *Proc. 3rd Int. Congr. Biochem., Brussels*, p. 356.

The Copper Content and Cytochrome Oxidase Activity of Tissues from Normal and Swayback Lambs

By J. McC. HOWELL* and A. N. DAVISON

Department of Pathology, Guy's Hospital Medical School, London, S.E. 1

(*Received* 3 *December* 1958)

Swayback is a non-febrile, ataxic disorder of newborn and young lambs. The pathological changes may include symmetrical cerebral demyelination, degeneration of tracts in the spinal cord and chromatolytic changes in the neurones of the brain-stem nuclei and ventral horn (Innes & Shearer, 1940). Both swayback and the related condition enzootic ataxia, which occurs in Australian lambs, are associated with a deficiency of copper. In Australia this deficiency is due to the low level of copper in the pasture (Bennetts & Beck, 1942). The 'swayback' pastures of this country contain adequate levels of copper (Allcroft, 1952), and it is thought that swayback is caused by a failure in the intermediary metabolism of copper in the ewe, or by the copper in the pasture being in an unavailable form (Hunter, Eden & Green, 1945).

* Present address: Department of Veterinary Pathology, Royal (Dick) School of Veterinary Studies, The University of Edinburgh.

Recently Gallagher, Judah & Rees (1956*a*, *b*) have examined the effects of copper deficiency in young rats and hens. It was found that although the copper content of liver, kidney and brain was reduced no demyelination was produced. The two major metabolic disturbances which resulted from this deficiency were loss of cytochrome oxidase activity and the suppression of phospholipid synthesis. This suggested that cytochrome oxidase activity may be reduced in swayback. The present investigation was undertaken to determine the copper content and cytochrome oxidase activity of tissues from normal and swayback animals with particular reference to the brain.

MATERIALS AND METHODS

Tissues were obtained as soon as possible after the animal had been killed and they were sent to us immediately by rail, either in a fresh state or packed in ice. Material was

collected from the abattoir as soon as possible after death. It was then stored at −20° until required. The control lambs had diseases other than swayback and were from areas where the incidence of swayback is very low. These animals were killed and the tissues sent to us immediately. In order to determine whether the tissues had lost cytochrome oxidase activity in the interval between killing and freezing, we dissected two lambs from an outbreak of swayback and the relevant tissues were placed on solid carbon dioxide within 10 min. of death.

The copper content of the tissues was determined by the method of Eden & Green (1940). Cytochrome oxidase activity was measured by the method of Schneider & Potter (1943) with ascorbic acid as substrate. Succinic dehydrogenase activity was determined by the method of Gallagher *et al.* (1956*a*). Dry weights were determined by drying in an oven at 110° to constant weight. Lipid extraction was carried out on several brain samples by homogenizing in chloroform–methanol (2:1, v/v) and, after filtration, total lipids were determined by weighing each sample to constant weight after evaporation of the solvent. It was found that there was no difference in the total lipid content of samples from normal- and swayback-lamb brains. All the swayback cases were of the delayed type, showing no gross cerebral cavitation.

RESULTS

Determinations of copper content were made on the following tissues: liver, kidney, frontal pole of the cerebrum, whole cerebellum, caudate nucleus and cerebral white matter, which consisted of the corpus callosum and centrum semi-ovale. Cytochrome oxidase activity was determined in the following tissues: liver, kidney, scrapings of frontal cortex, cerebellar peduncle and caudate nucleus.

The mean values for the copper content of tissues are presented in Table 1 together with values obtained by other workers. Wherever possible two samples were examined from each tissue.

There is a significant difference (P 0·01–0·001) between the values for copper content of grey matter (caudate nucleus) and white matter in the normal lamb.

In order to assess any loss of cytochrome oxidase activity as a result of imperfect storage of some samples, fresh brain and kidney were left for over a day at 25° in a moist atmosphere. There was no

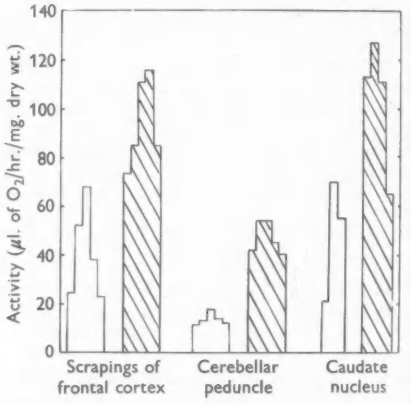


Fig. 1. Cytochrome oxidase in the brain of normal (shaded columns) and swayback lambs. Enzyme activity was determined as described in the text on five normal and five swayback lambs.

Table 1. *Copper contents of tissues of normal and swayback lambs*

Copper content is expressed as p.p.m. dry wt. (±S.E.M. and number of observations). Reference numbers (last column): 1, Present work; 2, Bennetts & Chapman (1937) on enzootic ataxia; 3, Schulz, Van der Merwe, van Rensburg & Swart (1951) on lamkruis, a condition closely resembling swayback; 4, Shearer, Innes & McDougall (1940); 5, Dick (1954).

Tissue	Copper content: Control	Copper content: Swayback	P	Reference no.
Liver	109·08±12·9 (12)	16·84±3·77 (9)	<0·0001	1
Liver	360–120	8–3	—	2
Liver	1000–100	24·9–2·2	—	3
Liver	—	14·8±1·9 (13)	—	4
Whole brain	30·2, 33·6	—	—	5
Whole brain	7·5±3·0 (30)	8·4±1·8 (9)	—	4
Kidney	13·45±1·7 (4)	15·79±1·5 (10)	0·4–0·3	1
Kidney	11·28	—	—	3
Kidney	14, 15·9	—	—	5
Brain:				
Frontal pole	15·53±0·71 (12)	5·37±1·02 (10)	<0·0001	1
Cerebellum	15·75±1·17 (12)	4·04±1·5 (11)	<0·0001	1
Caudate nucleus	19·26±3·9 (6)	1·4, 16	—	1
Cerebral white matter	8·15±1·81 (4)	1·71±0·57 (4)	0·01–0·0001	1

loss of cytochrome oxidase or succinic dehydrogenase activity. Furthermore, samples frozen immediately after death in no way differed from the tissues sent to us by rail on ice or in the fresh state. These results substantiate the observations of Greenstein, Werne, Eschenbrenner & Leuthardt (1944), who found that prolonged storage of guinea-pig tissues at $-20°$ or allowing them to stand for 4 hr. at room temperature did not result in loss of cytochrome oxidase activity.

Succinic dehydrogenase activity was determined in samples of brain from a swayback animal and of normal brain. The levels of the enzyme in normal brain did not differ from those in the swayback animals.

Cytochrome oxidase activity in brain samples from swayback and normal lambs is recorded in Fig. 1. These results show a clear difference in the activity of the enzyme in the brains of the two groups. The mean value for cytochrome oxidase activity in the liver of four swayback lambs is $42{\cdot}1 \pm 12{\cdot}3$ μl. of O_2/hr./mg. dry wt. and for five normal lambs is $63{\cdot}4 \pm 7{\cdot}85$ μl. of O_2/hr./mg. dry wt. The difference is on the borderline of significance (P 0·2–0·1). There was no difference in cytochrome oxidase activity of kidney from normal and swayback lambs.

DISCUSSION

Our results show that the copper content of swayback tissues is significantly lower than that of control tissues except for kidney. Our cases of swayback were delayed cases and the animals were fed away from the home farm before we received them. This food presumably had a normal copper content. We think that this may account for the high kidney figures. Shearer, Innes & McDougall (1940) found no loss of copper in the brain of swayback lambs. However, they were working in North Derbyshire and both the swayback and the non-swayback lambs that they examined were copper-deficient with liver-copper contents of $14{\cdot}8 \pm 0{\cdot}19$ and $18{\cdot}4 \pm 0{\cdot}72$ parts/million dry wt. respectively.

Cytochrome oxidase activity has been shown to be reduced in the brains of affected animals (Fig. 1). Since this enzyme was found to be stable at room temperature for at least a day and since the activity of succinic dehydrogenase was normal in the brains of the swayback lambs, it may be concluded that the fall in cytochrome oxidase activity was real and not due to post-mortem autolysis or to necrosis *in vivo*. There was also a small difference (P 0·1–0·05) between the cytochrome oxidase activity in the livers of normal and swayback lambs. The enzyme activity, however, remained unchanged in the kidney. These changes in cytochrome oxidase activity can be correlated with similar alterations in the copper content of brain and kidney. The liver is the main site of storage of copper and it is not therefore surprising that cytochrome oxidase activities remain high in this tissue even after 85 % copper depletion.

Hurst (1942) produced demyelination in sheep by the administration of potassium cyanide and in monkeys by the administration of potassium cyanide and sodium azide. Both these substances are cytochrome oxidase inhibitors. The absence of neurological lesions in the animals used in experiments of Gallagher *et al.* (1956*a*, *b*) may be related to the difficulty these workers experienced in reducing brain cytochrome oxidase activity. Hurst (1942) produced lesions only after chronic poisoning. Present evidence would suggest a chronic deficiency of copper and cytochrome oxidase activity in the swayback lambs.

Until recently the role of copper in tissue respiration at the level of cytochrome oxidase has not been clear (Gallagher *et al.* 1956*a*). The original suggestion of Keilin & Hartree (1938) that cytochrome oxidase contains copper has now, however, been confirmed (Wainio, Wende & Shimp, 1958), and Anderson & Tove (1958) have found that copper has a direct function in the synthesis of haem. Our present findings are consistent with this work and also with that of Gallagher *et al.* (1956*a*, *b*), although the part played by cytochrome oxidase in the demyelinating process in swayback remains to be investigated.

SUMMARY

1. The copper content of brain and liver from swayback lambs was significantly lower than normal.

2. Cytochrome oxidase activity in brain of swayback lambs was also significantly reduced.

3. The relationship between copper contents and cytochrome oxidase activity is discussed.

We are grateful to the following veterinary surgeons, who so kindly provided us with material: Dr J. C. Buxton, Messrs E. T. Davies, J. D. Paterson and P. J. N. Pinsent; and to Professor G. Payling Wright for his encouragement and interest in this work. This work was done while one of us (J.McC.H.) was on an Agricultural Research Council Scholarship.

REFERENCES

Allcroft, R. (1952). *Vet. Rec.* **64**, 17.

Anderson, R. L. & Tove, S. B. (1958). *Nature, Lond.*, **182**, 315.

Bennetts, H. W. & Beck, A. B. (1942). *Bull. Coun. Sci. Industr. Res. Aust.*, no. 147, 32.

Bennetts, H. W. & Chapman, F. E. (1937). *Aust. vet. J.* **13**, 138.

Dick, A. T. (1954). *Aust. J. agri. Res.* **5**, 511.
Eden, A. & Green, H. H. (1940). *Biochem. J.* **34**, 1202.
Gallagher, C. H., Judah, J. D. & Rees, K. R. (1956*a*). *Proc. Roy. Soc.* B, **145**, 134.
Gallagher, C. H., Judah, J. D. & Rees, K. R. (1956*b*). *Proc. Roy. Soc.* B, **145**, 195.
Greenstein, J. P., Werne, J., Eschenbrenner, A. B. & Leuthardt, F. M. (1944). *J. nat. Cancer Inst.* **5**, 55.
Hunter, A. H., Eden, A. & Green, H. H. (1945). *J. comp. Path.* **55**, 29.
Hurst, E. W. (1942). *Aust. J. exp. Biol. med. Sci.* **20**, 297.
Innes, J. R. M. & Shearer, G. D. (1940). *J. comp. Path.* **53**, 1.
Keilin, D. & Hartree, E. F. (1938). *Nature, Lond.*, **141**, 870.
Schneider, W. C. & Potter, V. R. (1943). *J. biol. Chem.* **149**, 217.
Schulz, K. C. A., Van der Merwe, P. K., van Rensburg, P. J. J. & Swart, J. S. (1951). *Onderstepoort J. vet. Res.* **25**, 35.
Shearer, G. D., Innes, J. R. M. & McDougall, E. I. (1940). *Vet. J.* **96**, 309.
Wainio, W. W., Wende, C. V. & Shimp, N. F. (1958). *Fed. Proc.* **17**, 330.